CONTRACT NUMBER DAMD17-92-C-2028

Analysis of Investigational Drugs in Biological Fluids - Method Development and Routine assay

FINAL REPORT - APPENDIX A
for the Period January 15, 1992 - January 14, 1996

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AD_____

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TITLE: Analysis of Investigational Drugs in Biological Fluids-Method Development and Routine Assay

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13. ABSTRACT (Maximum 200 words)

Using the procedures described in this report, we were able to work sequentially or simultaneously on eleven projects (1-WR 238,605, 2-halofantrine (and its metabolite), 3-WR 6026 (and its metabolites), 4-mefloquine (and its metabolite), 5-artelinic acid, 6-*p*-aminoheptanophenone (and related compounds), 7-primaquine (and its metabolite), 8-gentamicin and paromomycin, 9-pyridostigmine, 10-chloroquine (and its metabolites), and 11-a multiple drug interaction study in dog plasma for WR 238,605, mefloquine, chloroquine, quinine; doxycycline, and halofantrine with additional work on development and validation of LC/MS/MS methods for halofantrine (and its metabolite), WR 238,605) in terms of method development, validation, and characterization. We worked on demonstrating sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA).

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APPENDIX A

Method Validation Data

LABORATORY METHODOLOGY FOR WR 238,605 AS FREE BASE RAT PLASMA ASSAY,* STUDY REPORT 13, SUPPLEMENT I

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Altex Model 100A Solvent Delivery Module (Beckman Instruments Inc., Berkeley, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. WR 238,605 succinate (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK 73252, expiration date not available.
4. WR 6026 dihydrochloride (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK01845, expiration date not available.
5. Phosphoric acid (85%) (Fisher Scientific, Fair Lawn, NJ).
6. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
7. Sodium hydroxide (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Methyl *t*-butyl ether (Fisher Scientific, Fair Lawn, NJ).
10. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

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* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

C. ASSAY CONDITIONS

1. DETECTOR

Settings

Wavelength: excitation - 375 nm, emission - 480 nm
Sensitivity: high
Range: 4
Response: medium

Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

2. COLUMN

Phenomenex Silica, 5 µm particle size, 4.6 x 250 mm (Phenomenex Inc., Rancho Palos Verdes, CA).

3. SOLVENT SYSTEM

Combine and mix H_2O (2 L) + $(NH_4)_2HPO_4$ (20 mL of 1 M $(NH_4)_2HPO_4$) + CH_3CN (2 L).
Adjust apparent pH to 7.0 with 85% H_3PO_4.

4. FLOW RATE

1.2 ml/min

5. STOCK SOLUTIONS - Solutions were stored in a freezer and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

a. WR 238,605 (free base)

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
			Preparation date: 8/18/93		
Standard Curve	12.56	0.79695	50	methanol	200
			Preparation date: 6/21/93		
Precision	12.79	0.79695	50	methanol	204

*= Molecular weights of WR 238605 free base/WR 238605 succinate

b. WR 6026 internal standard.

Preparation date: 6/21/93

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal Std	11.16	1	100	methanol	112

2. WORKING SOLUTIONS - Store solution in a freezer and discard within 6 months.

a. WR 238605.

Preparation date: 8/18/93

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
Standard Curve	200	1:100	methanol	2.00
Standard Curve	2.00	1:10	methanol	0.200

Preparation date: 7/8/93

Precision	204	1:100	methanol	2.04
Precision	2.04	1:10	methanol	0.204

b. WR 6026 (Internal Standard).

Preparation date: 6/21/93

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
Internal Std.	112	1:4	methanol	27.9

7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.

a. WR 238,605 as free base - 5.1 min

b. WR 6026 as free base (Internal Standard) - 7.2 min

8. BLANK RAT PLASMA

Rat plasma (3.8% sodium citrate) Pel-Freez Biologicals, Rogers, AK.

9. INJECTION VOLUME

50-100 µl

10. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of $1/y_i$.

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum WR 238,605 (as free base) quantitation limit for the assay of rat plasma was based on interday and intraday precision results (Tables 2 and 3) and on standard curve calibrator results (Table 4).)

    Approximately 2 ng/ml WR 238,605 (free base) in plasma.

12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

    Plasma samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. The drug is spiked with a Hamilton syringe.

13. WISP OPERATING TEMPERATURE

    Room temperature.

D. SAMPLE STORAGE

All samples are to be kept frozen at -20°C before analysis and thawed at room temperature for preparation (within 30 min) and analysis.

E. SAMPLE PREPARATION

1. If frozen, thaw rat plasma sample at room temperature and vortex for 1 min. Pipet 0.2 ml of rat plasma into a 13 x 100 glass culture tube.

2. Spike standard curve samples with 00,* 0,** 1, 2, 4, 8, or 15 µl of 0.200 µg/ml WR 238605 working solution or 3, 5, 10, 20, or 40 µl of 2.00 µg/ml WR 238605 working solution to make a standard curve. Since 0.2 ml plasma samples are assayed, this procedure is equivalent to making standard curve samples with WR 238605 concentrations corresponding to 00, 0, 1.00, 2.00, 4.00, 8.00, 15.0, 30.0, 50.0, 100, 200 and 400 ng/ml. Vortex for 20 s.

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* 00 = Sample with no drug and no internal standard.
** 0 = Sample with no drug but with internal standard.

3. Add 20 µl of internal standard working solution (27.9 µg/ml WR 6026), except to 00 standard curve sample. Vortex 30 s.

4. Add 0.1 ml of 0.1 M NaOH. Vortex 30 s.

5. Add 3 ml of methyl *t*-butyl ether. Vortex for 1 min, twice, and centrifuge for 10 min at 3000 *g*.

6. Transfer the organic layer to a second 13 x 100 tube with a pasteur pipette and evaporate to dryness under nitrogen.

7. Reconstitute with 200 µl of 50% CH_3CN, transfer to WISP insert and inject onto column.

F. QUALITY CONTROL

1. Content and frequency of blanks

   No special blank was used except for the standard curve blank.

2. Pipette Calibration

   See ASOP 2C-1.1.

3. Balance Calibration

   See ASOP 2C-2.1.

G. RECOVERY

Assay recovery was assessed at four different concentrations by comparing the WR 238,605 (as free base) to internal standard peak height ratios in reference samples to the peak height ratios in plasma samples. Plasma (0.2 ml) and reference samples were spiked with corresponding amounts of WR 238,605 (as free base). Each plasma sample was prepared as described in "Sample Preparation" (Section E), except 2.5 ml of the extraction solvent was taken for evaporation (step 6), and the internal standard was added after the evaporation (step 6 not step 3) of the extraction solvent. The plasma samples were generated by spiking 0.2 ml of blank rat plasma with appropriate amounts of drug prior to addition of 0.1 N NaOH buffer. The reference samples (0.2 ml of blank rat plasma) were generated by spiking after the methyl *t*-butyl ether extraction with drug to the 2.5 ml of extraction solvent that was taken.

H. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 0.2 ml plasma specimens with WR 238,608 (as free base) working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	0.204	200	2.04
Low	15	0.204	200	15.3
Med.	5	2.04	200	51.0
Hi	20	2.04	200	204

I. RESULTS

1. STANDARD CURVE

   Chromatograms for each point in a representative standard curve for WR 238,605 as free base appear in Figure 1. Peak height ratios for these calibrators appear in Table 1.

2. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 2 and 3.

3. STUDY STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

   Results for this evaluation appears in Table 4

4. RECOVERY

   Results for this evaluation appear in Table 5.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR WR 238,605 (FREE BASE) RAT PLASMA ASSAY, STUDY REPORT 13, SUPPLEMENT NO. I

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
0.200	1.00	0.034	1.13
0.400	2.00	0.065	2.15
0.801	4.00	0.116	3.81
1.60	8.00	0.230	7.53
3.00	15.0	0.483	15.8
6.01	30.0	0.849	27.7
10.0	50.0	1.457	47.6
20.0	100	2.944	96.1
40.0	200	6.413	209
80.1	400	12.242	400

Regression equation:*** $y = 0.03060x - 0.00074$, $r^2 = 0.9987$

* Into 0.2 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

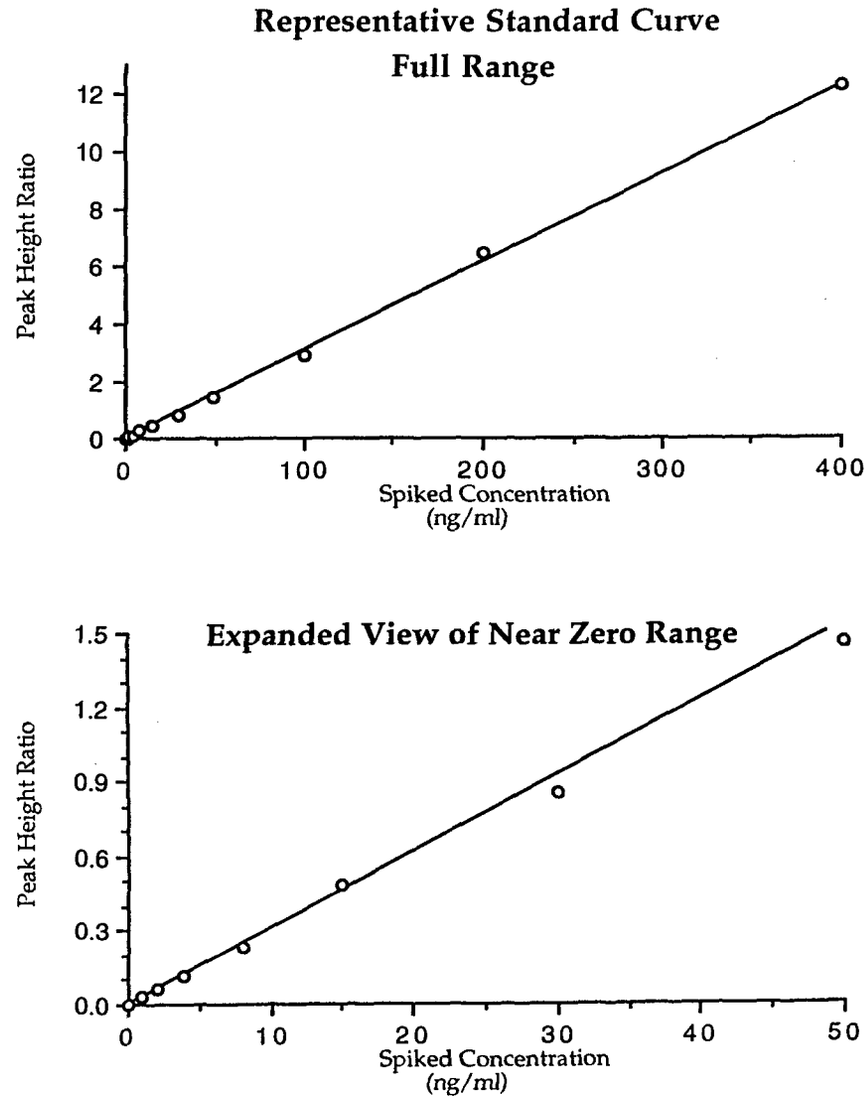

TABLE 2: INTERDAY PRECISION OF WR 238,605 FREE BASE RAT PLASMA ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3 Measured Concentrations* (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
2.04	2.13	2.09	1.94	2.05	0.100	4.88	0.654
15.3	14.8	14.9	16.4	15.4	0.896	5.83	0.436
51.0	54.6	45.9	55.5	52.0	5.30	10.2	1.96
204	230	218	239	229	10.5	4.60	12.3

TABLE 3: INTRADAY PRECISION OF WR 238,605 FREE BASE RAT PLASMA ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6 Measured Concentrations (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
2.04	2.35	2.07	1.86	2.21	1.75	2.03	2.05	0.220	10.8	0.245
15.3	15.0	14.9	14.6	14.3	14.8	15.1	14.8	0.293	1.98	-3.38
51.0	43.4	44.2	46.8	46.6	42.3	46.8	45.0	1.98	4.39	-11.7
204	215	210	212	216	216	216	214	2.56	1.20	4.98

* Measured concentrations are averages of two analyses.

TABLE 4: STUDY STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS FOR WR 238,605 RAT PLASMA ASSAY, STUDY REPORT 13 SUPPLEMENT I

Spiked Concentration (ng/ml)	n	Mean (ng/ml)	Standard Deviation (ng/ml)	Percent C.V.	Percent Deviation
1.00	3	1.25	0.188	15.0	25.3
2.00	3	2.05	0.111	5.40	2.33
4.00	3	3.89	0.067	1.71	-2.83
8.00	3	7.75	0.276	3.56	-3.13
15.0	3	15.4	0.551	3.57	2.89
30.0	3	27.5	0.473	1.72	-8.22
50.0	3	46.0	2.86	6.21	-8.00
100	3	93.2	2.51	2.70	-6.80
200	3	210	1.73	0.82	5.00
400	3	405	6.81	1.679	1.33

TABLE 5: RECOVERY OF WR 238,605 FROM RAT PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	(ng/ml)	PEAK HEIGHT RATIO REFERENCE	PLASMA	MEAN PERCENT RECOVERY
WR 238,605					
1	X Low	5.00	0.087	0.052	63.9
2			0.075	0.048	
3			0.071	0.049	
Mean (± SD)			0.078 ± 0.008	0.050 ± 0.002	
1	Low	15.0	0.535	0.379	69.5
2			0.469	0.336	
3			0.507	0.335	
Mean (± SD)			0.504 ± 0.033	0.350 ± 0.025	
1	Medium	50.0	1.774	1.183	68.7
2			1.772	1.123	
3			1.698	1.299	
Mean (± SD)			1.748 ± 0.043	1.202 ± 0.089	
1	High	200	7.513	5.135	63.5
2			7.421	4.799	
3			7.932	4.577	
Mean (± SD)			7.622 ± 0.272	4.837 ± 0.281	
AVERAGE MEAN RECOVERY = 66.4					

TABLE 5: RECOVERY OF WR 238,605 FREE BASE FROM DOG PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	(ng/ml)	PEAK HEIGHT RATIO REFERENCE	PLASMA	MEAN PERCENT RECOVERY
WR 238,605 free base					
1	X Low	5.00	0.069	0.048	70.4
2			0.066	0.045	
3			0.068	0.050	
Mean (± SD)			0.068 ± 0.002	0.048 ± 0.003	
1	Low	15.0	0.515	0.371	72.7
2			0.505	0.366	
3			0.484	0.356	
Mean (± SD)			0.501 ± 0.016	0.364 ± 0.008	
1	Medium	50.0	1.614	1.105	63.3
2			1.721	1.008	
3			1.566	0.989	
Mean (± SD)			1.634 ± 0.079	1.034 ± 0.062	
1	High	200	7.368	4.342	59.7
2			8.223	4.882	
3			BC	4.749	
Mean (± SD)			7.796 ± 0.605	4.658 ± 0.281	
AVERAGE MEAN PERCENT RECOVERY = 66.5					

LABORATORY METHODOLOGY FOR WR 238,605 AS FREE BASE DOG PLASMA ASSAY,* STUDY REPORT 13, SUPPLEMENT II

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Altex Model 100A Solvent Delivery Module (Beckman Instruments Inc., Berkeley, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. WR 238,605 succinate (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK 73252, expiration date not available.
4. WR 6026 dihydrochloride (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK01845, expiration date not available.
5. Phosphoric acid (85%) (Fisher Scientific, Fair Lawn, NJ).
6. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
7. Sodium hydroxide (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Methyl *t*-butyl ether (Fisher Scientific, Fair Lawn, NJ).
10. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

C. ASSAY CONDITIONS

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* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

1. DETECTOR

   Settings

   Wavelength: excitation - 375 nm, emission - 480 nm
   Sensitivity: high
   Range: 4
   Response: medium

   Lamp

   Ushio xenon, type UXL-155-LCA(S-LC)

2. COLUMN

   Phenomenex Silica, 5 µm particle size, 4.6 x 250 mm (Phenomenex Inc., Rancho Palos Verdes, CA).

3. SOLVENT SYSTEM

   CH_3CN/H_2O (50:50, v/v) and 5 mM $(NH_4)_2HPO_4$, pH = 7.0 (adjusted with 85% H_3PO_4).

4. FLOW RATE

   1.2 ml/min

5. STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

a. WR 238,605 (free base)

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
			Preparation date: 1/6/94		
Standard Curve	12.56	0.79695	50	methanol	200
			Preparation date: 6/21/93		
Precision	12.79	0.79695	50	methanol	204

*= Molecular weights of WR 238605 free base/WR 238605 succinate

b. WR 6026 internal standard.

Preparation date: 1/6/94

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal Std	11.16	1	100	methanol	112

2. WORKING SOLUTIONS - Store solution at 4°C and discard within 6 months.

a. WR 238605.

Preparation date: 1/6/94

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
Standard Curve	200	1:100	methanol	2.00
Precision	204	1:100	methanol	2.04

Preparation date: day of use

Standard Curve	2.00	1:10	methanol	0.200
Precision	2.04	1:10	methanol	0.204

b. WR 6026 (Internal Standard).

Preparation date: 6/21/93

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
Internal Std.	112	1:4	methanol	27.9

7. RETENTION TIMES (subject to change depending on temperature and column performance).

a. WR 238,605 as free base - 5.1 min

b. WR 6026 as free base (Internal Standard) - 7.2 min

8. BLANK DOG PLASMA

Dog plasma (3.8% sodium citrate, heparin) Pel-Freez Biologicals, Rogers, AK.

9. INJECTION VOLUME

50-100 µl

10. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression.

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum quantitation limit was determined as the WR 238,605 (free base) standard curve concentration at which the signal to noise ratio was at least 3 to 1.)

    1.00 ng/ml WR 238,605 (free base) in plasma.

12. SAMPLE VOLUME MEASUREMENT

    Plasma sample volumes were measured with a variable volume Eppendorf pipetter.

13. WISP OPERATING TEMPERATURE

    Room temperature.

D. SAMPLE STORAGE

All samples are to be kept frozen at -20°C before analysis and thawed at room temperature for preparation (within 30 min) and analysis.

E. SAMPLE PREPARATION

1. Pipet 0.2 ml of dog plasma into a 13 x 100 culture tube.

2. Spike standard curve samples with 00, 0, 1, 2, 4, 8, or 15 µl of 0.200 µg/ml WR 238605 working solution or 3, 5, 10, 20, or 40 µl of 2.00 µg/ml WR 238605 working solution to make a standard curve. Since 0.2 ml plasma samples are assayed, this procedure is equivalent to making standard curve samples with WR 238605 concentrations corresponding to 00, 0, 1.00, 2.00, 4.00, 8.00, 15.0, 30.0, 50.0, 100, 200 and 400 ng/ml. Vortex for 20 s.

3 Thaw clinical samples at room temperature.

4. Vortex for 1 min.

5. Add 20 µl of internal standard working solution (27.9 µg/ml WR 6026). Vortex 30 s.

6. Add add 0.1 ml of 0.1 M NaOH. Vortex 30 s.

7. Add 3 ml of methyl *t*-butyl ether. Vortex for 2 min and centrifuge for 10 min at 3000 *g*.

8. Transfer the organic layer to a second 13 x 100 tube and evaporate to dryness under nitrogen.

9. Reconstitute with 200 µl of 50% CH_3CN and inject onto column.

F. QUALITY CONTROL

1. Content and frequency of blanks

   No special blank was used except for the standard curve blank.

2. Pipette Calibration

   See ASOP 2C-1.1.

3. Balance Calibration

   See ASOP 2C-2.1.

G. RECOVERY

Assay recovery was assessed at four different concentrations by comparing the WR 238,605 (as free base) to internal standard peak height ratios in a reference sample to the peak height ratios in plasma. Plasma (0.2 ml) and reference samples were spiked with corresponding amounts of WR 238,605 (as free base). Each plasma sample was prepared as described in "Sample Preparation" (Section E), except samples were centrifuged for 10 minutes (step 7), 2.5 ml of sample was taken for evaporation (step 8), and the internal standard was added after the evaporation (step 8). The reference samples were spiked prior to extraction (step 5) with drug and after evaporation (step 8) with internal standard.

H. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 0.2 ml plasma specimens with WR 238,608 (as free base) working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	0.204	200	2.04
Low	15	0.204	200	15.3
Med.	5	2.04	200	51.0
Hi	20	2.04	200	204

I. RESULTS

1. STANDARD CURVE

   Chromatograms for each point in a representative standard curve for WR 238,605 as free base appear in Figure 1. Peak height ratios for these calibrators appear in Table 1.

2. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 2 and 3.

3. STUDY STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

   Results for this evaluation appears in Table 4

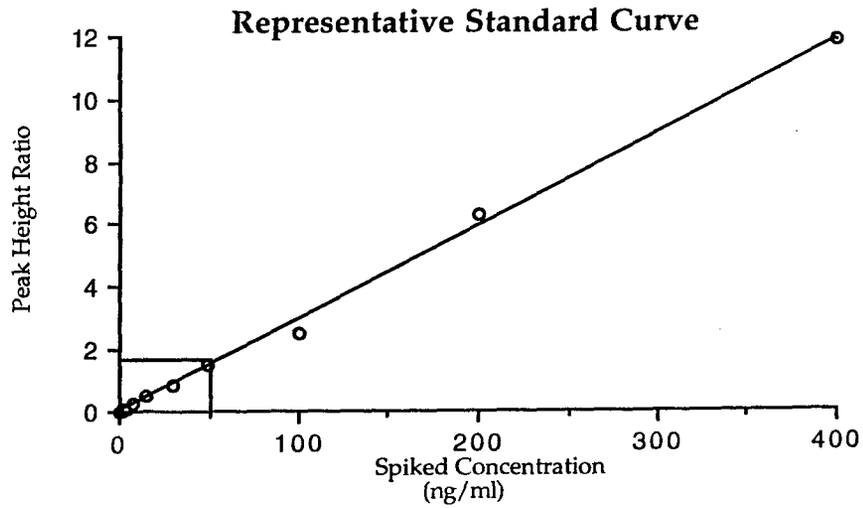
4. RECOVERY

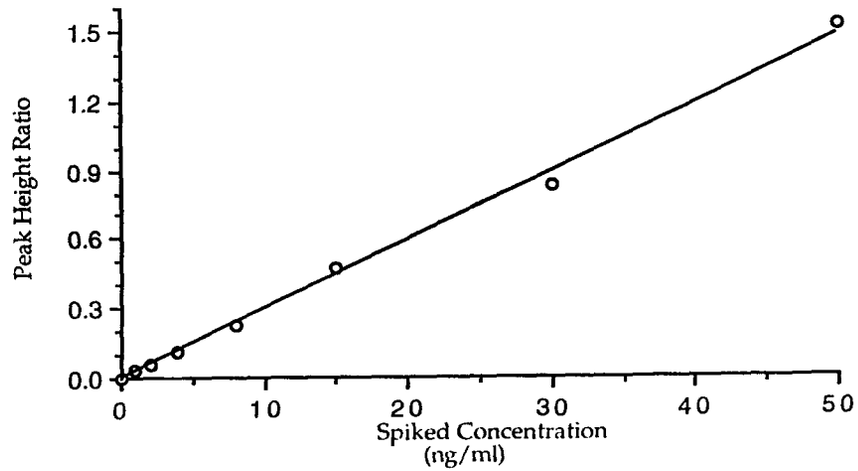
   Results for this evaluation appear in Table 5.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR WR 238,605 (FREE BASE) DOG PLASMA ASSAY, STUDY REPORT 13, SUPPLEMENT II

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
0.200	1.00	0.031	1.13
0.400	2.00	0.061	2.15
0.800	4.00	0.107	3.72
1.60	8.00	0.219	7.55
3.00	15.0	0.466	16.0
6.01	30.0	0.826	28.3
10.0	50.0	1.527	52.2
20.0	100	2.501	85.5
40.0	200	6.253	214
80.0	400	11.817	404

Regression equation:***
$y = 0.0293x - 0.00198$, $r^2 = 0.9951$





* Into 0.2 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = 1/y.

TABLE 2: INTERDAY PRECISION OF WR 238,605 FREE BASE DOG PLASMA ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3 Measured Concentrations* (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
2.04	2.44	2.43	2.38	2.41	0.033	1.38	18.3
15.3	14.3	14.1	14.4	14.3	0.076	0.54	-6.75
51.0	54.4	51.5	59.0	55.1	3.56	6.46	8.07
204	222	208	221	218	6.54	3.01	6.62

TABLE 3: INTRADAY PRECISION OF WR 238,605 FREE BASE DOG PLASMA ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6 Measured Concentrations (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
2.04	2.34	2.04	2.21	2.01	1.91	2.14	2.11	0.154	7.31	3.35
15.3	14.9	14.4	13.7	16	13.1	14.6	14.5	1.00	6.93	-5.56
51.0	50.4	60.7	46.7	51.9	47.1	49.1	51.0	5.15	10.1	-0.033
204	217	200	213	201	192	199	204	9.42	4.62	-0.163

* Measured concentrations are averages of two analyses.

TABLE 4: STUDY STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS FOR WR 238,605 DOG PLASMA ASSAY, STUDY REPORT 13 SUPPLEMENT

Spiked Concentration (ng/ml)	n	Mean (ng/ml)	Standard Deviation (ng/ml)	Percent C.V.	Percent Deviation
1.00	4	1.22	0.0818	6.72	21.8
2.00	4	2.09	0.0920	4.40	4.50
4.00	4	3.73	0.135	3.62	-6.81
8.00	4	7.47	0.199	2.66	-6.59
15.0	4	14.4	1.11	7.75	-4.33
30.0	4	27.5	1.35	4.93	-8.50
50.0	4	52.1	0.926	1.78	4.10
100	4	97.8	8.39	8.58	-2.18
200	4	211	6.95	3.30	5.38
400	4	396	9.26	2.34	-1.13

TABLE 5: RECOVERY OF WR 238,605 FREE BASE FROM DOG PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	(ng/ml)	PEAK HEIGHT RATIO REFERENCE	PLASMA	MEAN PERCENT RECOVERY
WR 238,605 free base					
1	X Low	5.00	0.069	0.048	70.4
2			0.066	0.045	
3			0.068	0.050	
Mean (± SD)			0.068 ± 0.002	0.048 ± 0.003	
1	Low	15.0	0.515	0.371	72.7
2			0.505	0.366	
3			0.484	0.356	
Mean (± SD)			0.501 ± 0.016	0.364 ± 0.008	
1	Medium	50.0	1.614	1.105	63.3
2			1.721	1.008	
3			1.566	0.989	
Mean (± SD)			1.634 ± 0.079	1.034 ± 0.062	
1	High	200	7.368	4.342	59.7
2			8.223	4.882	
3			BC	4.749	
Mean (± SD)			7.796 ± 0.605	4.658 ± 0.281	
AVERAGE MEAN PERCENT RECOVERY = 66.5					

LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460 (AS FREE BASES) BLOOD AND PLASMA ASSAY,* STUDY REPORT 17

presented in mid term report

* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460 (AS FREE BASES) BLOOD AND PLASMA ASSAY,* STUDY REPORT 17B

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. LC-600 Shimadzu Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade unless otherwise specified.
2. All chemicals are reagent grade unless otherwise specified.
3. Halofantrine hydrochloride, WR 171,669, bottle no. BB 43807 (Walter Reed Army Institute of Research, Washington D.C.).
3. WR 178,460, bottle no. BK 21070 (Walter Reed Army Institute of Research, Washington D.C.).
4. WR 122,455 , bottle no. AX 26839 (Walter Reed Army Institute of Research, Washington D.C.).
5. Methanol Optima Grade (Fisher Scientific, Fair Lawn, NJ).
6. Acetonitrile (Fisher Scientific, Fair Lawn, NJ).
7. Methyl *t* butyl ether (Baxter, Burdick & Jackson, Muskegon, MI).
8. Sodium hydroxide (Mallinckrodt Co., Paris, KY).
9. Water (deionized by Nanopure II, Barnstead Co., Boston, MA).
10. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).

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* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

C. ASSAY CONDITIONS

1. DETECTOR

   Settings

   Wavelength; excitation - 300 nm, emission - 375 nm
   Sensitivity - high and Range - 32

   Lamp: Ushio xenon, type UXL-155-LCA(S-LC)

2. COLUMN

   Phenomenex Silica, 5 µm particle size, 4.6 x 250 mm (Phenomenex Inc., Rancho Palos Verdes, CA) or equivalent.

3. SOLVENT SYSTEM

   CH_3OH/H_2O (80:20, v/v) + 5 mM $(NH_4)_2HPO_4$ (final concentration) (Optima methanol)

4. FLOW RATE

   1.0 ml/min

5. REPRESENTATIVE STOCK SOLUTIONS - Stock solutions of halofantrine, its metabolite and the internal standard were kept at -20°C and covered in aluminum foil. Stock solutions of halofantrine and its metabolite were checked for deterioration by HPLC comparison of neat injections to newly made solutions (solutions are discarded when a more than 10% change in the absolute peak height is observed or by 6 months after the preparation date).

   a. Halofantrine (free base) for interday plasma precision.

Prep date: 9/7/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.54	0.942	50	methanol	104
Control	5.44	0.942	50.6	methanol	101

*= Molecular weights of halofantrine free base/halofantrine hydrochloride

   b. WR 178,460 (free base) for interday plasma precision.

Prep date: 9/7/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.62	0.924	50	methanol	104
Control	5.62	0.924	50	methanol	104

*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

c. WR 122,455 - Internal standard for interday plasma precision.

Prep date: 7/13/94

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	5.10	1	25	methanol	204

6. REPRESENTATIVE WORKING SOLUTIONS - Solutions were stored in a -20°C freezer, covered in aluminum foil, and discarded when stock solutions were discarded or by 6 months after the preparation date).

a. Mixed halofantrine and WR 178,460 (as free bases) solutions.

Low concentration solution. Combine 0.500 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 100 ml for interday plasma precision.

Prep date: 9/13/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	0.500	100	methanol	0.520
Standard Curve (WR 178,460)	104	0.500	100	methanol	0.520

High concentration solution. Combine 2.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 100 ml for interday plasma precision.

Prep date: 9/13/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	2.00	100	methanol	2.08
Standard Curve (WR 178,460)	104	2.00	100	methanol	2.08

Low concentration solution. Combine 0.500 ml each of halofantrine and WR 178,460 (as free bases) stock control curve solutions and q.s. to 100 ml for interday plasma precision.

Prep date: 9/13/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Control (Halofantrine)	101	0.500	100	methanol	0.505
Control (WR 178,460)	104	0.500	100	methanol	0.520

High concentration solution. Combine 2.00 ml each of halofantrine and WR 178,460 (as free bases) stock control solutions and q.s. to 100 ml for interday plasma precision.

Prep date: 9/13/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Control (Halofantrine)	101	2.00	100	methanol	2.02
Control (WR 178,460)	104	2.00	100	methanol	2.08

b. WR 122,455 - Internal standard for interday plasma precision.

Prep date: 9/7/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (part)	QS Volume (part)	Solvent	Conc. (µg/ml)
Internal std.	204	0.5	200	methanol	0.510

7. RETENTION TIMES (subject to change depending on temperature and column performance).

   a. Halofantrine (free base) - 8 min
   b. WR 178,460 (free base) - 11 min
   c. WR 122,455 (Internal Standard) - 14 min

8. BLANK PLASMA AND BLOOD

   Human plasma and blood (CPD or CPDA-1 as anticoagulant) is obtained from the San Francisco Irwin Memorial Blood Bank.

9. INJECTION VOLUME: Samples that are expected to have high halofantrine or WR 178,460 concentrations (i.e. high standard curve calibrators, high concentration control samples, and sponsor samples shown or expected to be near C_{peak}) are injected at the low end of the volume range.

   50-150 µl

10. QUANTITATION

    By peak height ratio of drug peak and metabolite peak relative to internal standard peak. Standard curves are calculated by non weighted linear regression and are split into low and high range curves.

11. MINIMUM QUANTITATION LIMITS OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the

assay of human and plasma were based on the interday and intraday low point validation results and on standard curve calibrator results.)

2.08 ng/ml halofantrine (free base) in plasma.
2.08 ng/ml WR 178,460 (free base) in plasma.

1.02 ng/ml halofantrine (free base) in blood.
0.964 ng/ml WR 178,460 (free base) in blood.

12. SAMPLE VOLUME MEASUREMENT

Plasma sample volumes were measured with a 200 µl or a 1000 µl Gilson Pipetman. Blood sample volumes were measured with Eppendorf pipettes.

13. WISP OPERATING TEMPERATURE

Room temperature.

14. SAMPLE EVAPORATION

Extracted samples are evaporated in a N-EVAP® Model 112 (Organomatic Assoc, Inc., S. Berlin, MA) by passing N_2 over the sample. The samples do not sit in water during evaporation.

D. SAMPLE STORAGE

All samples were kept frozen at -70°C before analysis and thawed and held on ice until prepared for analysis, unless specified otherwise.

E. SAMPLE PREPARATION

PLASMA SAMPLES

1. Pipet 0.5 ml of plasma into a 13x100 mm silanized tube on ice.
2. Spike standard curve samples on ice as shown in Section G "Generation of Standard Curve Calibrators" and vortex for 30 s.
3. Add 150 µl of internal standard working solution (WR 122,455, 0.51 µg/ml) on ice. Vortex for 30 s.
4. Add 1 ml acetonitrile. Vortex for 1 min and repeat. Centrifuge for 10 min at 3000 *g*.
5. Transfer supernatant to 16x125 mm silanized tube and evaporate to 0.5 ml.
6. Add 0.5 ml water and 50 µl of 0.1 N NaOH. Vortex for 30 s.

7. Add 5 ml methyl-*t*-butyl ether. Vortex for 1 min and repeat. Centrifuge for 10 min at 3000 *g*.
8. Freeze mixture in dry ice/methanol bath. Transfer organic phase to silanized 13x100 mm tube and begin evaporation.
9. Repeat steps 7 and 8, transferring organic phase to same 13x100 mm tube and evaporate to dryness.
10. Reconstitute residue with 200 µl of 80% methanol containing 0.001% HCl. Vortex for 2 min.
11. Transfer to silanized glass WISP inserts and inject onto column.

BLOOD SAMPLES

1. Pipet 0.5 ml of blood into a 13x100 mm silanized tube on ice.
2. Follow step 2 as in plasma sample preparation. Vortex for 20 s, and let stand on ice for 1 h.
3. Add (while samples are on ice) 0.5 ml water. Vortex for 10 s.
4. Sonicate for 10 min in water bath.
5. Add 50 µl of internal standard working solution (WR 122,455, 0.51 µg/ml). Vortex for 20 s.
6. Add 2 ml acetonitrile. Vortex for 2.5 min. Centrifuge for 15 min at 3000 *g*.
7. Follow steps 5-11 as in plasma sample preparation.

F. QUALITY CONTROL

1. CONTENT AND FREQUENCY OF BLANKS

   A blank plasma or blood sample was prepared as described in "Sample Preparation" and assayed at least once for each standard curve in precision assays.

2. PIPETTE CALIBRATION

   See SOP 2C-1.1.

3. BALANCE CALIBRATION

   See SOP 2C-2.1

G. GENERATION OF STANDARD CURVE CALIBRATORS

A representative example of the generation of standard curve calibrators is shown in the table below. Spike blank plasma standard curve samples on ice with halofantrine and WR 178,460 (as free bases)

mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 0.500 ml plasma samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

Generation of Halofantrine and WR 178,460 (as free bases) Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (ng/ml)
00*	0	0	0	0
0**	0	0	0	0
1	2	0.520	1.04	2.08
2	4	0.520	2.08	4.16
3	8	0.520	4.16	8.32
4	4	2.08	8.32	16.6
5	8	2.08	16.64	33.3
6	16	2.08	33.28	66.6
7	32	2.08	66.56	133
8	64	2.08	133.12	266

H. GENERATION OF PRECISION SAMPLES

A representative example of the generation of precision controls is shown in the table below. Samples for precision analysis were prepared by spiking 0.5 ml plasma or blood specimens with control working solutions to make the halofantrine and WR 178,460 (as free bases) concentrations shown.

Generation of Halofantrine Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	4	0.505	0.5	4.04
Low	10	0.505	0.5	10.1
Med.	10	2.02	0.5	40.4
Hi	32	2.02	0.5	129

Generation of WR 178,460 Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	4	0.520	0.5	4.16
Low	10	0.520	0.5	10.4
Med.	10	2.08	0.5	41.6
Hi	32	2.08	0.5	133

* 00 = Sample with no drug and no internal standard.
** 0 = Sample with no drug but with internal standard.

I. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at four different concentrations by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in plasma or blood. Plasma or blood (0.5 ml) samples were spiked with halofantrine and WR 178,460 (as free bases) then prepared as described above in "Sample Preparation," except the internal standard was added after the extracts were evaporated (step 10). Reference samples were generated by spiking reconstitution solvent with drug and internal standard.

J. GENERATION OF STABILITY SAMPLES

System stability samples were generated in the same way as precision control samples.

Bench top stability samples were generated in the same way as precision control samples at low and high concentrations.

The effect of repeated freeze and thaw cycles on stabilities of halofantrine and WR 178,460 (as free bases) in human plasma and blood samples was determined as follows: Spiked (low and high concentrations) pooled biological sample were subjected to five thaw/freeze cycles. For each cycle, a duplicate set of thaw/freeze samples (0.5 ml) was generated at each concentration. The study is run with the following procedure:

a. Prepare high and low concentration samples labeled H-1, H-2 ... H-5, and L-1, L-2 ... L-5, in duplicate.

b. Store all samples until frozen at the specified temperature.

c. Repeatedly thaw and refreeze samples according to the following table. Thaw as if for sample preparation to room temperature. Let thawed samples stand at room temperature for 1 h.

Cycle	Keep these samples in freezer	Thaw these samples
1	1	2, 3, 4, 5
2	1, 2	3, 4, 5
3	1, 2, 3	4, 5
4	1, 2, 3, 4	5
5	1, 2, 3, 4, 5	none

d. Following Cycle 5, take out all of the samples, thaw to room temperature, and assay the samples with a standard curve.

K. VALIDATION RESULTS

1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figures 4 and 5. Peak height ratios for these calibrators appear in Tables 1A-B and 2A-B. Statistical parameters of plasma interday precision standard curve calibrators appear in Table 3A and of blood interday and intraday precision standard curve calibrators appear in Table 3B.

2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 4A-B and 5A-B.

3. LLOQ

Results for this evaluation appear in Tables 6A-B.

4. RECOVERY

Results for this evaluation appear in Tables 7A-B.

6. STABILITY

a. System Stability: Results appear in Tables 8A-B.

b. Long Term Stability:* Pooled plasma and blood samples spiked with halofantrine and WR 178,460 (as free bases) at four different concentrations were mixed on a rotator for one hour. The resulting samples were divided into 0.5 ml fractions, placed in culture tubes and stored in the freezer at -80°C until assayed for stability. Samples were assayed according to the method described in Study Report No. 4 dated Aug. 23, 1985 and titled "Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite (WR 178,460) in Blood and Plasma." Results appear in Table 9A-B.

c. Bench Top Stability: Results appear in Tables 10A-B.

d. Freeze/Thaw Stability: Results appear in Tables 11A-B.

8. BLIND SAMPLE ANALYSIS

Results appear in 12A-B for blood.

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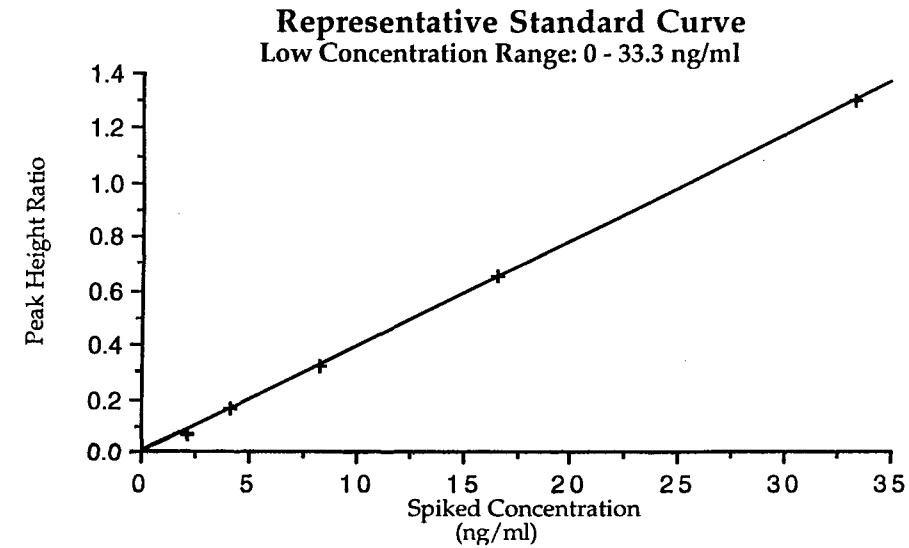
* Samples were assayed according to the method described in Study Report No. 4 dated Aug. 23, 1985 and titled "Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite (WR 178,460) in Blood and Plasma."

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR HALOFANTRINE (FREE BASE) PLASMA ASSAY, STUDY REPORT 17B

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
1.04	2.08	0.072	1.88[a]
2.08	4.16	0.169	4.35[a]
4.16	8.32	0.321	8.22[a]
8.32	16.6	0.654	16.7[a]
16.64	33.3	1.303	33.3[a]
33.28	66.6	2.830	67.6[b]
66.56	133	5.525	131[b]
133.12	266	11.279	267[b]

Regression equations:***
[a] $y = 0.039228x - 0.001608$, $r^2 = 0.9999$ (Low Range: 0 - 33.3 ng/ml)
[b] $y = 0.042393x - 0.035294$, $r^2 = 0.9998$ (High Range: 0 - 266 ng/ml)

* Into 0.5 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level (free base) concentrations, two standard curves were constructed from the same set of standard curve data points.



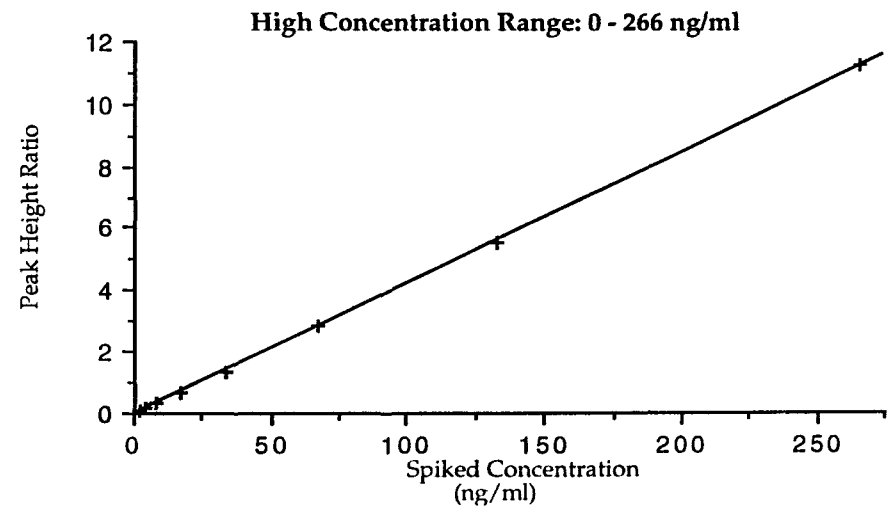


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR WR 178,460 (FREE BASE) PLASMA ASSAY, STUDY REPORT 17B

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
1.04	2.08	0.125	1.97[a]
2.08	4.16	0.289	4.62[a]
4.16	8.32	0.523	8.41[a]
8.32	16.6	0.992	16.0[a]
16.64	33.3	2.075	33.5[a]
33.28	66.6	4.311	67.4[b]
66.56	133	8.375	131[b]
133.12	266	17.159	267[b]

Regression equations:***
[a] $y = 0.061782x + 0.003593$, $r^2 = 0.9992$ (Low Range: 0 - 33.3 ng/ml)
[b] $y = 0.064353x - 0.029110$, $r^2 = 0.9998$ (High Range: 0 - 266 ng/ml)

* Into 0.5 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level (free base) concentrations, two standard curves were constructed from the same set of standard curve data points.

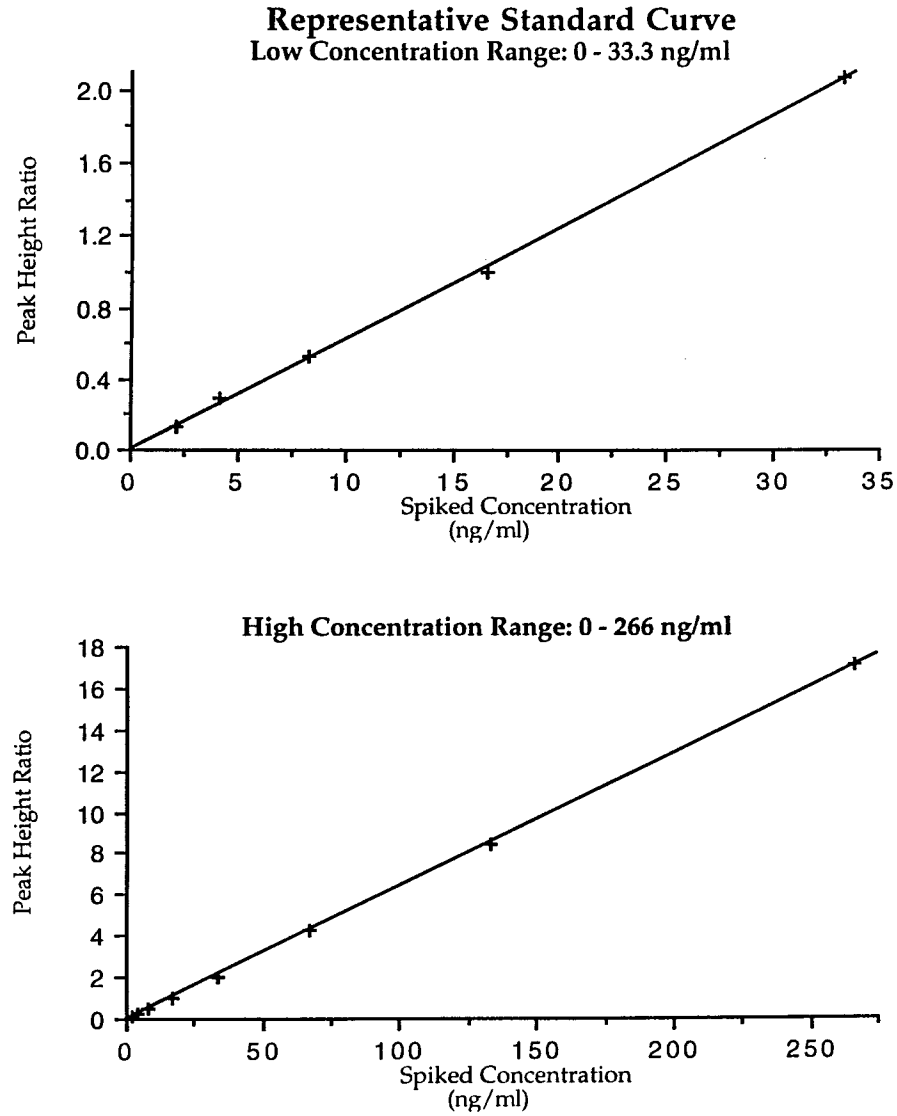
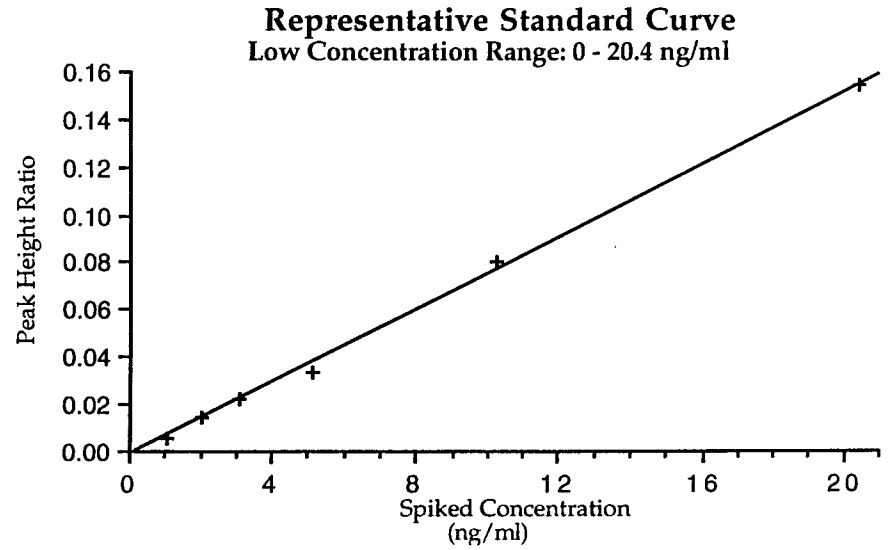


TABLE 2A: REPRESENTATIVE STANDARD CURVE FOR HALOFANTRINE (FREE BASE) BLOOD ASSAY, STUDY REPORT 17B

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
0.511	1.02	0.006	0.978[a]
1.02	2.04	0.014	2.02[a]
1.53	3.07	0.022	3.07[a]
2.56	5.11	0.034	4.63[a]
5.11	10.2	0.080	10.6[a]
10.2	20.4	0.154	20.3[a]
20.4	40.8	0.297	41.0[b]
40.8	81.6	0.582	79.8[b]
61.2	122	0.849	116[b]
122	245	1.819	248[b]

Regression equations:***
[a] $y = 0.007658x - 0.00149$, $r^2 = 0.9985$ (Low Range: 0 - 20.4 ng/ml)
[b] $y = 0.007341x - 0.00391$, $r^2 = 0.9991$ (High Range: 0 - 245 ng/ml)

* Into 0.5 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level (free base) concentrations, two standard curves were constructed from the same set of standard curve data points.



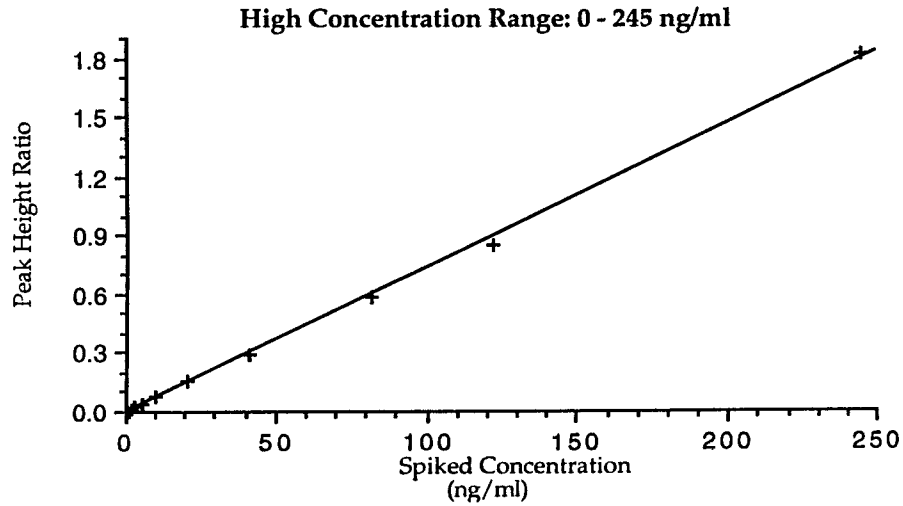
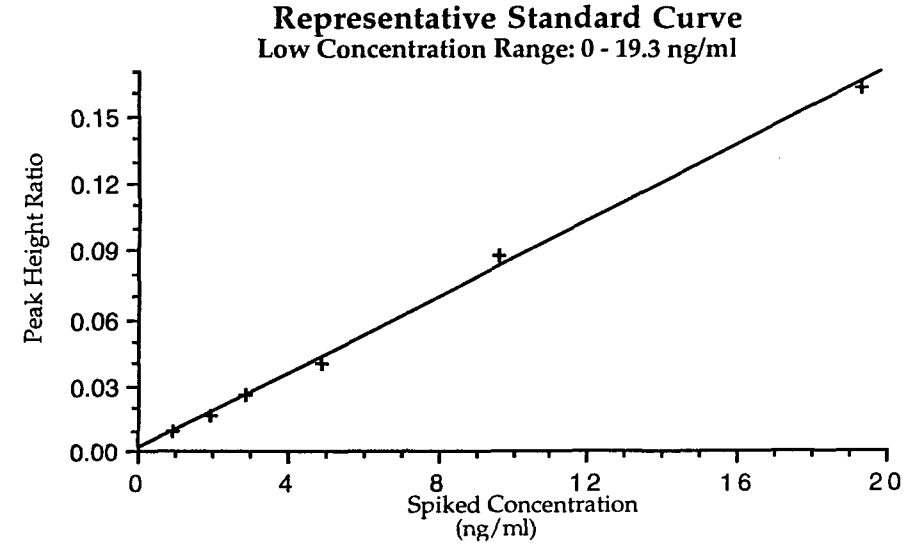


TABLE 2B: REPRESENTATIVE STANDARD CURVE FOR WR 178,460 (FREE BASE) BLOOD ASSAY, STUDY REPORT 17B

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
0.482	0.964	0.010	1.02[a]
0.964	1.93	0.017	1.84[a]
1.45	2.89	0.026	2.90[a]
2.41	4.82	0.041	4.67[a]
4.82	9.64	0.088	10.2[a]
9.64	19.3	0.163	19.1[a]
19.3	38.6	0.331	38.6[b]
38.6	77.2	0.653	76.0[b]
57.9	116	0.974	113[b]
116	232	2.013	234[b]

Regression equations:***
[a] $y = 0.008483x + 0.001366$, $r^2 = 0.9983$ (Low Range: 0 - 19.3 ng/ml)
[b] $y = 0.008621x - 0.00212$, $r^2 = 0.9997$ (High Range: 0 - 232 ng/ml)

* Into 0.5 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level (free base) concentrations, two standard curves were constructed from the same set of standard curve data points.



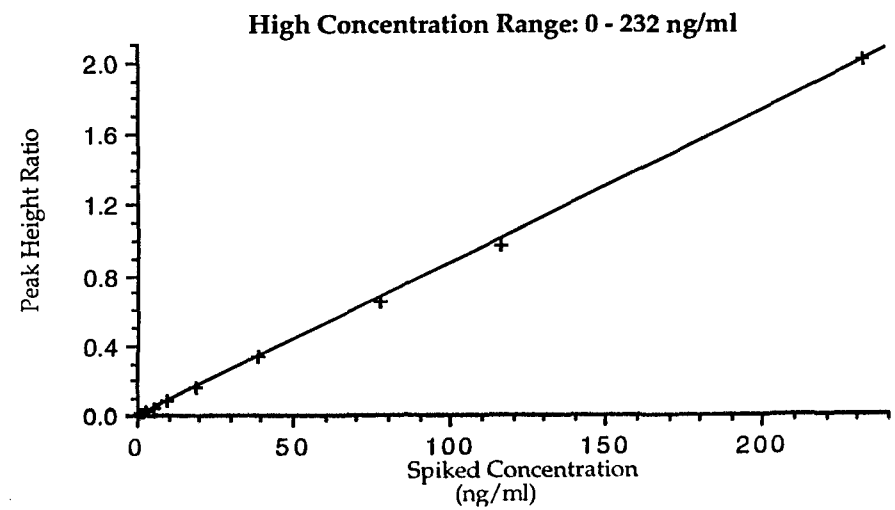


TABLE 3A: INTERDAY PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS FOR HALOFANTRINE PLASMA ASSAY, SR 17B

Spiked Concentration (ng/ml)	n	Mean (ng/ml)	Standard Deviation (ng/ml)	Percent C.V.	Percent Deviation
		HALOFANTRINE			
2.08	6	1.81	0.217	12.0	-13.0
4.16	6	4.06	0.306	7.55	-2.44
8.32	6	8.39	0.791	9.42	0.89
16.6	6	17.2	1.51	8.76	3.71
33.3	6	33.0	0.574	1.74	-0.89
66.6	6	63.7	3.00	4.71	-4.38
133	6	131	7.36	5.60	-1.17
266	6	268	4.12	1.54	0.61
		WR 178,460			
2.08	5	2.02	0.267	13.3	-3.06
4.16	6	4.25	0.441	10.4	-3.06
8.32	6	8.45	0.538	6.37	2.06
16.6	6	16.7	0.889	5.32	1.55
33.3	6	33.2	0.330	0.99	0.62
66.6	6	65.5	2.02	3.09	-0.27
133	6	132	7.69	5.81	-1.60
266	6	267	4.00	1.50	-0.55

TABLE 3B: PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS FOR HALOFANTRINE BLOOD ASSAY, SR 17B

Spiked Concentration (ng/ml)	n	Mean (ng/ml)	Standard Deviation (ng/ml)	Percent C.V.	Percent Deviation
		HALOFANTRINE			
1.02	6	1.14	0.124	10.9	11.9
2.04	7	2.08	0.117	5.62	2.19
3.07	7	2.95	0.242	8.20	-3.79
5.11	7	4.73	0.234	4.96	-7.42
10.2	7	10.7	0.354	3.33	4.45
20.4	7	20.3	0.157	0.773	-0.608
40.8	7	40.2	2.86	7.13	-1.56
81.6	7	79.7	3.53	4.43	-2.37
122	7	121	6.42	5.32	-1.21
245	7	246	3.29	1.34	0.604
		WR 178,460			
0.964	7	0.994	0.068	6.79	3.13
1.93	7	1.96	0.193	9.83	1.54
2.89	7	2.94	0.177	6.03	1.75
4.89	7	4.88	0.222	4.54	1.24
9.64	7	9.65	0.478	4.95	0.092
19.3	7	19.3	0.197	1.02	-0.163
38.6	7	38.5	1.56	4.04	-0.245
77.2	7	75.3	1.58	2.09	-2.44
116	7	116	2.83	2.45	-0.107
232	7	233	1.76	0.756	0.321

TABLE 4A: PRECISION OF HALOFANTRINE HUMAN PLASMA ASSAY

Inter-Run Precision Halofantrine Free Base

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 4.04	10.1	40.4	129
		Measured Concentrations (ng/mL)			
1	1	4.84	10.0	43.1	155
	2	4.58	9.65	40.7	126
2	1	4.27	9.26	39.5	122
	2	4.66	11.2	46.2	116
3	1	3.64	10.7	40.1	143
	2	3.24	9.67	41.6	142
4	1	3.53	10.1	42.5	133
	2	3.33	10.7	38.6	137
5	1	3.31	9.61	33.3	124
	2	3.99	8.68	38.1	115
6	1	3.88	bc	38.8	135
	2	4.29	10.1	38.2	150
n		12	11	12	12
Mean		3.96	9.97	40.1	133
S.D.		0.563	0.715	3.2	12.9
Percent C.V.		14.2	7.17	8.00	9.69
Percent R.E.		-1.87	-1.25	-0.825	3.31

Intra-Run Precision Pyridostigmine Cation

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 2.04	10.2	61.2	102
		Measured Concentrations (ng/mL)			
7	1	1.56	9.80	51.4	80.5
	2	1.73	9.94	52.1	87.0
	3	1.94	9.77	52.9	93.7
	4	1.94	10.4	48.1	83.6
	5	1.89	11.1	53.1	89.4
	6	1.98	9.72	49.7	90.9
n		6	6	6	6
Mean		1.84	10.1	51.2	87.5
S.D.		0.161	0.54	1.96	4.86
Percent C.V.		8.75	5.35	3.83	5.55
Percent R.E.		-9.80	-1.00	-16.3	-14.2

bc = chromatogram unacceptable.

TABLE 4B: PRECISION OF HALOFANTRINE HUMAN PLASMA ASSAY

Inter-Run Precision WR 178,460 Free Base

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 4.16	10.4	41.6	133
		Measured Concentrations (ng/mL)			
1	1	4.48	10.8	43.3	153
	2	4.60	10.4	41.9	131
2	1	4.18	10.6	42.3	126
	2	bc	11.4	47.1	126
3	1	3.18	10.3	42.0	146
	2	4.27	10.5	41.7	143
4	1	bc	10.8	44.0	136
	2	4.49	10.2	41.0	138
5	1	4.00	bc	34.7	124
	2	4.16	9.69	38.1	116
6	1	4.62	bc	42.1	138
	2	5.26	10.3	40.2	150
n		10	10	12	12
Mean		4.32	10.5	41.5	136
S.D.		0.532	0.441	3.04	11.1
Percent C.V.		12.3	4.19	7.32	8.20
Percent R.E.		3.91	1.04	-0.149	1.94

Intra-Run Precision WR 178,460 Free Base

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 1.93	9.64	57.9	96.5
		Measured Concentrations (ng/mL)			
7	1	2.13	10.1	56.8	92.9
	2	1.98	10.2	56.1	93.1
	3	1.98	9.95	56.9	98.1
	4	1.93	9.89	54.6	94.0
	5	1.98	10.1	55.4	96.6
	6	2.30	9.71	55.5	98.1
n		6	6	6	6
Mean		2.05	10	55.9	95.5
S.D.		0.142	0.194	0.906	2.45
Percent C.V.		6.95	1.94	1.62	2.56
Percent R.E.		6.11	3.78	-3.49	-1.08

bc = chromatogram unacceptable.

TABLE 5A: PRECISION OF HALOFANTRINE HUMAN BLOOD ASSAY

Inter-Run Precision Halofantrine Free Base

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 4.16	10.4	41.6	133
		Measured Concentrations (ng/mL)			
1	1	1.71	10.8	60.4	103
	2	2.01	8.35	59.2	101
2	1	2.43	11.7	60.7	105
	2	2.11	10.4	66.0	90.8
3	1	2.15	11.0	62.9	106
	2	2.02	11.2	62.0	99.2
4	1	2.19	11.0	60.1	102
	2	1.91	9.82	59.7	104
5	1	1.66	10.7	52.5	87.5
	2	1.96	12.6	59.9	93.4
6	1	1.91	9.85	62.8	89.9
	2	bc	11.2	58.1	104
n		11	12	12	12
Mean		2.01	10.7	60.4	98.8
S.D.		0.218	1.06	3.25	6.59
Percent C.V.		10.8	9.91	5.38	6.67
Percent R.E.		-1.69	5.08	-1.38	-3.12

Intra-Run Precision Halofantrine Free Base

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 2.04	10.2	61.2	102
		Measured Concentrations (ng/mL)			
7	1	1.86	10.4	53.4	90.8
	2	2.00	7.68	58.4	107
	3	2.00	9.75	53.3	89.5
	4	1.58	bc	64.0	103
	5	2.14	7.68	57.4	109
	6	1.72	11.0	46.2	113
n		6	5	6	6
Mean		1.88	9.30	55.5	102
S.D.		0.206	1.55	6.00	9.77
Percent C.V.		11.0	16.7	10.8	9.58
Percent R.E.		-7.84	-8.82	-9.31	0

bc = chromatogram unacceptable.

TABLE 5B: PRECISION OF HALOFANTRINE HUMAN BLOOD ASSAY

Inter-Run Precision WR 178,460 Free Base

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 1.93	9.64	57.9	96.5
		Measured Concentrations (ng/mL)			
1	1	1.60	9.26	60.4	99.5
	2	1.85	9.26	58.5	95.9
2	1	1.69	9.38	58.7	98.8
	2	1.81	9.63	57.9	94.0
3	1	1.84	10.2	58.4	97.8
	2	1.73	9.98	57.2	96.4
4	1	2.02	9.80	57.1	96.0
	2	2.02	9.31	58.4	99.2
5	1	1.85	9.35	53.3	88.6
	2	1.97	9.84	56.9	92.9
6	1	2.00	9.55	58.4	93.6
	2	bc	10.0	57.2	93.5
n		11	12	12	12
Mean		1.93	9.63	57.7	95.5
S.D.		0.142	0.331	1.67	3.17
Percent C.V.		7.67	3.44	2.90	3.32
Percent R.E.		-4.04	-0.0765	-0.351	-1.02

Intra-Run Precision WR 178,460 Free Base

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 2.04	10.2	61.2	102
		Measured Concentrations (ng/mL)			
7	1	1.70	10.0	54.3	92.2
	2	1.83	9.20	55.0	97.6
	3	1.83	9.83	51.5	93.1
	4	1.83	bc	58.1	97.9
	5	1.83	9.33	56.8	99.3
	6	1.95	10.1	55.9	99.5
n					
Mean		1.83	9.68	55.3	96.6
S.D.		0.079	0.389	2.29	3.15
Percent C.V.		4.33	4.02	4.14	3.26
Percent R.E.		-5.34	0.400	-4.53	0.100

bc = chromatogram unacceptable.

TABLE 6A: LOW POINT VALIDATION OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) HUMAN PLASMA ASSAY

Spiked Concentration	HALOFANTRINE (free base) (2.08 ng/ml)		WR 178,460 (free base) (2.08 ng/ml)	
	Measured Concentrations (ng/ml)			
	Interday	Intraday	Interday	Intraday
	1.98	2.06	1.86	2.13
	1.53	1.51	b.c.	2.16
	1.79	1.80	2.06	2.22
	1.88	2.48	1.97	2.04
	1.59	1.99	1.75	2.01
	2.08	bc	2.45	b.c.
	1.81	1.97	2.02	2.11
Mean				
Standard Deviation	0.217	0.36	0.267	0.09
Percent CV	12.0	18.2	13.3	4.07
Percent Error	-13.0	-5.67	-3.06	1.52

TABLE 6B: LOW POINT VALIDATION OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) HUMAN BLOOD ASSAY

Spiked Concentration	HALOFANTRINE (free base) (1.02 ng/ml)		WR 178,460 (free base) (0.964 ng/ml)	
	Measured Concentrations (ng/ml)			
	Interday	Intraday	Interday	Intraday
	1.10	1.28	1.09	b.c.
	b.c.	1.01	0.928	0.798
	0.978	1.01	1.02	0.923
	1.19	b.c.	0.927	0.923
	1.07	1.14	0.971	1.05
	1.34	1.14	0.951	0.923
Mean	1.14	1.12	0.980	0.923
Standard Deviation	0.138	0.112	0.062	0.088
Percent CV	12.2	10.1	6.35	9.57
Percent Error	11.4	9.45	1.70	-4.28

bc = unacceptable chromatogram.

TABLE 7A: RECOVERIES OF HALOFANTRINE AND WR 178,460 FROM HUMAN PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	(ng/ml)	PEAK HEIGHT RATIO SOLVENT	PLASMA	MEAN PERCENT RECOVERY
HALOFANTRINE					
1	X Low	2.04	0.011	0.005	55.2
2			0.007	0.005	
3			0.011	0.006	
Mean (± SD)			0.010 ±0.002	0.005 ±0.001	
1	Low	10.2	0.074	0.035	52.4
2			0.064	0.023	
3			0.068	0.050	
Mean (± SD)			0.069 ±0.005	0.036 ±0.014	
1	Medium	61.2	0.416	0.193	35.7
2			0.421	0.060	
3			0.415	0.194	
Mean (± SD)			0.417 ±0.003	0.149 ±0.077	
1	High	102	0.685	0.278	42.5
2			0.701	0.342	
3			0.694	0.263	
Mean (± SD)			0.693 ±0.008	0.294 ±0.042	
AVERAGE =					46.4
WR 178,460					
1	X-Low	1.93	0.011	0.011	105.9
2			0.010	0.010	
3			0.013	0.015	
Mean (± SD)			0.011 ±0.002	0.012 ±0.003	
1	Low	9.64	0.075	0.060	78.4
2			0.077	0.056	
3			0.079	0.065	
Mean (± SD)			0.077 ±0.002	0.060 ±0.005	
1	Medium	57.9	0.454	0.335	72.2
2			0.458	0.316	
3			0.469	0.346	
Mean (± SD)			0.460 ±0.008	0.332 ±0.015	
1	High	96.5	0.748	0.546	75.1
2			0.774	0.636	
3			0.748	0.522	
Mean (± SD)			0.757 ±0.015	0.568 ±0.060	
AVERAGE =					82.9

TABLE 7B: RECOVERIES OF HALOFANTRINE AND WR 178,460 FROM HUMAN BLOOD

SAMPLE ID	SPIKED CONCENTRATION Range	(ng/ml)	PEAK HEIGHT RATIO SOLVENT	PLASMA	MEAN PERCENT RECOVERY
HALOFANTRINE					
1	X-Low	2.04	0.062	0.043	80.0
2			bc	0.054	
3			0.063	0.053	
Mean (± SD)			0.063 ±0.001	0.050 ±0.006	
1	Low	10.2	0.382	0.345	79.2
2			0.399	0.269	
3			0.389	0.313	
Mean (± SD)			0.390 ±0.009	0.309 ±0.038	
1	Medium	61.2	2.263	1.842	82.0
2			2.357	2.027	
3			2.466	1.944	
Mean (± SD)			2.362 ±0.102	1.938 ±0.093	
1	High	102	3.954	3.227	81.9
2			3.766	3.340	
3			3.989	3.020	
Mean (± SD)			3.903 ±0.120	3.196 ±0.162	
AVERAGE =					80.8
WR 178,460					
1	X-Low	1.93	0.087	0.078	88.5
2			bc	0.074	
3			0.084	0.075	
Mean (± SD)			0.086 ±0.002	0.076 ±0.002	
1	Low	9.64	0.436	0.379	85.8
2			0.416	0.357	
3			0.416	0.352	
Mean (± SD)			0.423 ±0.012	0.363 ±0.014	
1	Medium	57.9	2.512	2.193	85.8
2			2.607	2.257	
3			2.708	2.266	
Mean (± SD)			2.609 ±0.098	2.239 ±0.040	
1	High	96.5	4.457	3.971	90.4
2			4.191	3.980	
3			4.287	3.737	
Mean (± SD)			4.312 ±0.135	3.896 ±0.138	
AVERAGE =					87.6

bc = unacceptable chromatogram.

TABLE 8A: SYSTEM STABILITY IN PREPARED HUMAN PLASMA

Concentration for Prepared Biological Samples Stored at Room Temperature

Halofantrine (Free Base)

	CONCENTRATION# (ng/ml)			
Spiked Concentration:	2.04	10.2	61.2	102
TIME STORED				
0 day	2.08	10.8	60.0	98.4
1 day	2.21	11.4	63.1	106

WR 178,460 (Free Base)

Spiked Concentration:	1.93	9.64	57.9	96.5
TIME STORED				
0 day	2.09	9.81	56.7	94.8
1 day	2.26	10.2	60.3	101

TABLE 8B: SYSTEM STABILITY IN PREPARED HUMAN BLOOD

Concentration for Prepared Biological Samples Stored at Room Temperature

Halofantrine (Free Base)

	CONCENTRATION# (ng/ml)			
Spiked Concentration:	2.04	10.2	61.2	102
TIME STORED				
0 day	2.07	9.72	59.6	102
1 day	2.07	9.85	60.1	96.7

WR 178,460 (Free Base)

Spiked Concentration:	1.93	9.64	57.9	96.5
TIME STORED				
0 day	1.86	9.63	56.2	95.2
1 day	2.08	9.33	56.9	93.7

#Measured concentrations are averages of two analyses.

TABLE 9A: STABILITY OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) IN PLASMA#

HALOFANTRINE (FREE BASE) CONCENTRATION IN PLASMA STORED AT -80°C

	CONCENTRATION (ng/ml)			
Spiked Concentration:	4.50	10.8	28.8	63.0
DAYS STORED				
0	4.68	9.96	26.9	59.0
1	4.76	11.2	26.2	56.9
2	4.20	9.76	25.8	57.6
29	4.85	10.8	24.5	56.2
60	3.99	10.3	30.1	63.5
92	3.68	9.41	21.4	45.6
112	3.81	8.91	23.4	55.2
126	4.02	10.2	27.3	55.4
MEAN	4.25	10.1	25.7	56.2

WR 178,460 (FREE BASE) CONCENTRATION IN PLASMA STORED AT -80°C

	CONCENTRATION (ng/ml)			
Spiked Concentration:	7.00	16.8	44.8	98.0
DAYS STORED				
0	7.78	17.4	50.7	101
1	6.30	17.3	47.8	104
2	7.73	21.6	51.2	108
29	8.42	19.7	46.7	104
60	6.91	16.6	48.4	100
92	5.34	15.3	45.0	95.4
112	7.16	16.6	44.6	106
126	7.40	18.1	45.7	86.4
MEAN	7.13	17.8	47.5	101

Concentrations are means of multiple (usu. 3) analyses.

Table taken from Project Status Report No. 7, dated Dec. 23, 1987 from data obtained according to the method described in Study Report No. 4, dated Aug. 23, 1985 and titled "Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite (WR 178,460) in Blood and Plasma."

TABLE 9B: STABILITY OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) IN BLOOD#

HALOFANTRINE (FREE BASE) CONCENTRATION IN BLOOD STORED AT -80°C

	CONCENTRATION (ng/ml)			
Spiked Concentration:	4.50	10.8	28.8	63.0
DAYS STORED				
0	5.57	12.0	31.2	65.0
1	5.29	11.2	28.9	61.4
4	4.62	9.98	28.5	63.4
29	3.72	9.25	24.4	51.7
61	4.47	10.7	27.9	60.3
90	4.86	11.5	28.2	45.9
110	5.30	13.9	30.5	55.5
128	3.99	11.6	27.3	60.1
MEAN	4.73	11.3	28.4	57.9

WR 178,460 (FREE BASE) CONCENTRATION IN BLOOD STORED AT -80°C

	CONCENTRATION (ng/ml)			
Spiked Concentration:	7.00	16.8	44.8	98.0
DAYS STORED				
0	8.62	19.1	50.6	102
1	10.1	17.6	39.7	107
4	6.73	17.7	47.4	101
29	7.84	21.2	49.5	108
61	6.66	17.1	44.2	94.2
90	8.84	20.0	46.1	69.2
110	7.77	16.5	44.9	85.6
128	5.80	18.2	45.1	90.5
MEAN	7.80	18.4	45.9	94.7

Concentrations are means of multiple (usu. 3) analyses.

Table taken from Project Status Report No. 7, dated Dec. 23, 1987 from data obtained according to the method described in Study Report No. 4, dated Aug. 23, 1985 and titled "Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite (WR 178,460) in Blood and Plasma."

TABLE 10A: BENCH TOP STABILITY OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) IN SPIKED HUMAN PLASMA #

	HALOFANTRINE (free base)		WR 178,460 (free base)	
Spiked Concentration	Low Concentration (10.2 ng/ml)	High Concentration (102 ng/ml)	Low Concentration (9.64 ng/ml)	High Concentration (96.5 ng/ml)
HOURS				
0	10.4	103	9.97	93.9
1	11.1	103	9.63	94.3
2	10.4	103	9.53	96.9
4	10.5	107	9.63	96.2

TABLE 10B: BENCH TOP STABILITY OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) IN SPIKED HUMAN BLOOD ¥

	HALOFANTRINE (free base)		WR 178,460 (free base)	
Spiked Concentration	Low Concentration (10.2 ng/ml)	High Concentration (102 ng/ml)	Low Concentration (9.64 ng/ml)	High Concentration (96.5 ng/ml)
HOURS				
0	10.4	101	9.73	96.1
1	9.57	92.4	8.99	93.4
2	10.3	103	10.1	94.5
4	10.3	102	9.34	95.5

Measured concentrations are averages of two analyses.
¥ Measured concentrations are averages of three analyses.

TABLE 11A: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON HALOFANTRINE AND WR 178,460 (AS FREE BASES) SPIKED HUMAN PLASMA SAMPLES#

	HALOFANTRINE (free base)		WR 178,460 (free base)	
	Low Concentration	High Concentration	Low Concentration	High Concentration
Spiked Concentration	(10.2 ng/ml)	(102 ng/ml)	(9.64 ng/ml)	(96.5 ng/ml)
Cycle				
1	10.9	103	9.89	94.3
2	10.8	103	9.73	95.6
3	10.6	102	9.68	94.4
4	11.6	112	9.03	98.8
5	11.1	102	8.93	93.0

TABLE 11B: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON HALOFANTRINE AND WR 178,460 (AS FREE BASES) SPIKED HUMAN BLOOD SAMPLES@ ¥

	HALOFANTRINE (free base)		WR 178,460 (free base)	
	Low Concentration	High Concentration	Low Concentration	High Concentration
Spiked Concentration	(10.2 ng/ml)	(102 ng/ml)	(9.64 ng/ml)	(96.5 ng/ml)
Cycle				
1	10.4	90.4	9.24	89.1
2	10.1	93.4	9.35	91.3
3	10.0	95.0	9.39	89.9
4	9.74	92.5	9.27	90.8
5	9.92	99.4	9.12	92.6

Measured concentrations are averages of two analyses.
@ Individually spiked samples.
¥ Measured concentrations are averages of three analyses.

TABLE 12A: ACCURACY OF HALOFANTRINE (FREE BASE) HUMAN BLOOD ASSAY (BLIND STUDY RESULTS)

Sample Number	Spiked Level (ng/ml)	Measured Level# (ng/ml)	Statistics (ng/ml)
1	0	*	Mean =
12		*	SD =
13		*	Percent CV =
24		*	Percent Bias =
2	2.04	1.82	Mean = 2.05
11		2.05	SD = 0.170
15		2.09	Percent CV = 8.31
22		2.23	Percent Bias = 0.368
3	20.4	20.0	Mean = 19.8
10		20.0	SD = 0.300
14		19.6	Percent CV = 1.52
23		19.4	Percent Bias = -3.19
4	40.8	40.8	Mean = 39.3
8		36.5	SD = 2.19
17		41.3	Percent CV = 5.58
21		38.7	Percent Bias = -3.62
5	102	90.9	Mean = 93.6
9		92.9	SD = 2.24
16		96.1	Percent CV = 2.39
20		94.6	Percent Bias = -8.21
6	183.6	171	Mean = 173
7		172	SD = 2.63
18		173	Percent CV = 1.52
19		177	Percent Bias = -5.64

Measured concentrations are averages of three analyses.
* = Below assay sensitivity.

TABLE 12B: ACCURACY OF WR 178,460 (FREE BASE) HUMAN BLOOD ASSAY (BLIND STUDY RESULTS)

Sample Number	Spiked Level (ng/ml)	Measured Level# (ng/ml)	Statistics (ng/ml)
6	0	0.893	Mean = 1.03
7		1.05	SD = 0.121
18		0.982	Percent CV = 11.8
19		1.18	Percent Bias =
5	1.97	2.52	Mean = 2.635
9		2.50	SD = 0.150
16		2.71	Percent CV = 5.70
20		2.81	Percent Bias = 33.8
4	19.7	20.2	Mean = 19.8
8		19.3	SD = 0.45
17		19.6	Percent CV = 2.27
21		20.2	Percent Bias = 0.635
3	39.4	38.2	Mean = 38.4
10		37.6	SD = 0.665
14		38.8	Percent CV = 1.73
23		39.1	Percent Bias = -2.47
2	98.6	95.2	Mean = 98.3
11		96.3	SD = 3.12
15		102	Percent CV = 3.18
22		99.7	Percent Bias = -0.304
1	177.5	178	Mean = 181
12		178	SD = 3.79
13		186	Percent CV = 2.10
24		180	Percent Bias = 1.69

Measured concentrations are averages of three analyses.

LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460 AS FREE BASES IN RAT PERFUSATE PRECIPITATION ASSAY,* STUDY REPORT 17B, SUPPLEMENT I

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Halofantrine hydrochloride (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BB43807, expiration date not available.
4. WR 178,460 (hydrochloride) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK21070, expiration date not available.
5. WR 122,455 (internal standard) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. AX26839, expiration date not available.
6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

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* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

C. ASSAY CONDITIONS

1. DETECTOR

   Settings

   Wavelength: excitation - 300 nm, emission - 375 nm
   Sensitivity: high
   Range: 4
   Response: medium

   Lamp

   Ushio xenon, type UXL-155-LCA(S-LC)

2. COLUMN

   Axxiom Silica, 5 µm particle size, 4.6 x 250 mm
   (Richard Scientific, Novato, CA).

3. SOLVENT SYSTEM

   Combine and mix H_2O (800 m L) + $(NH_4)_2HPO_4$ (20 mL of 1 M $(NH_4)_2HPO_4$) + CH_3OH (3200 L).

4. FLOW RATE

   1.0 ml/min

5. STOCK SOLUTIONS - Solutions were stored in the xx°C freezer (refrigerator) and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

   A. Stock Solutions

   i. HALOFANTRINE - (Halofantrine Hydrochloride free base concentrations).

Prep date: 3/1/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.60	0.942	50.6	methanol	104
Precision	6.24	0.942	50.0	methanol	118

*= Molecular weights of halofantrine free base/halofantrine hydrochloride

ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride, free base concentrations).

Prep date: 3/1/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.60	0.924	50.6	methanol	102
Precision	6.03	0.924	52.0	methanol	107

*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

iii. WR 122,455 (Internal Standard) - (WRAIR, Washington, D.C.).

Prep date: 12/14/93

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	5.20	1	25	methanol	208

B. Working Solutions

i. HALOFANTRINE AND WR 178,460 MIXED WORKING SOLUTIONS (free base concentrations).

a. HIGH CONCENTRATION WORKING SOLUTION - Combine 10.0 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions.

Prep date: 3/22/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	10.0	20.0	methanol	52.0
Precision (Halofantrine)	118	10.0	20.0	methanol	59.0
Standard Curve (WR 178,460)	102	10.0	20.0	methanol	51.0
Precision (WR 178,460)	107	10.0	20.0	methanol	53.5

b. LOW CONCENTRATION WORKING SOLUTION - Combine 1.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 10 ml.

Prep date: 3/22/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	1.00	10.0	methanol	10.4
Precision (Halofantrine)	118	1.00	10.0	methanol	11.8
Standard Curve (WR 178,460)	102	1.00	10.0	methanol	10.2
Precision (WR 178,460)	107	1.00	10.0	methanol	10.7

ii. WR 122,455 - Internal standard.

Prep date: 3/22/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	208	0.500	10.5	methanol	9.90

7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.

   a. Halofantrine - 6.2 min
   b. WR 178,460 - 8.6 min
   c. WR 122,455 (Internal Standard) - 10.3 min

8. BLANK RAT PERFUSATE

   Supplied by WRAIR.

9. INJECTION VOLUME

   5-20 µl

10. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of $1/y_i$.

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat perfusate were based on the interday and intraday low point validation results (Table 2) and on standard curve calibrator results (Tables 1 and 4).)

    5.20 µg/ml halofantrine (free base).

    5.10 µg/ml WR 178,460 (free base).

12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

    Plasma samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. The drug is spiked with a Hamilton syringe.

13. WISP OPERATING TEMPERATURE

    Room temperature.

D. SAMPLE STORAGE

All samples are to be kept frozen at -70°C before analysis and thawed at room temperature for preparation (within 30 min) and analysis.

E. SAMPLE PREPARATION

1. Pipet 100 µl rat perfusate samples into 13 X 100 silanized tubes.
2. Add 40 µl of 9.90 µg/ml WR 122,455 internal standard solution. Vortex for 30s.
3. Add 0.4 ml CH_3CN. Vortex 1 min.
4. Centrifuge 10 min at 3000 *g*.
5. Transfer supernatant to silanized inserts and inject onto column.

F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 0.1 ml perfusate samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

Generation of Halofantrine Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (µg)	Standard Curve Sample Nominal Concentration (µg/ml)
00*	0	10.4	0	0
0**	0	10.4	0	0
1	5	10.4	52.0	0.520
2	10	10.4	104	1.04
3	20	10.4	208	2.08
4	8	52.0	416	4.16
5	16	52.0	832	8.32
6	32	52.0	1664	16.64
7	64	52.0	3328	33.28

Generation of WR 178,460 Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (µg)	Standard Curve Sample Nominal Concentration (µg/ml)
00	0	10.2	0	0
0	0	10.2	0	0
1	5	10.2	51.0	0.510
2	10	10.2	102	1.02
3	20	10.2	204	2.04
4	8	51.0	408	4.08
5	16	51.0	816	8.16
6	32	51.0	1632	16.32
7	64	51.0	3264	32.64

G. QUALITY CONTROL

1. Content and frequency of blanks
   No special blank was used except for the standard curve blank.

2. Pipette Calibration
   See ASOP 2C-1.1.

3. Balance Calibration
   See ASOP 2C-2.1.

H. RECOVERY

Assay recovery was assessed at three different concentrations by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in perfusate samples. The perfusate samples were generated by

* 00 = Sample with no drug and no internal standard.
** 0 = Sample with no drug but with internal standard.

spiking 100 µl of blank rat perfusate with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation) prior to addition of acetonitrile precipitant. Each perfusate sample was prepared as described in "Sample Preparation" (Section E), except the internal standard was added to the transfered supernatant (after step 5, not in step 3). The reference samples were generated by spiking water (100 µl in silanized tubes) with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). Reference samples were prepared by vortexing in acetonitrile, then internal standard, and transfering to silanized inserts for injection onto the HPLC column.

I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 100 µl perfusate specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Halofantrine Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (µg/ml)
Low	10	11.8	100	1.18
Med.	10	59.0	100	5.90
Hi	40	59.0	100	23.6

Generation of WR 178,460 Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (µg/ml)
Low	10	10.7	100	1.07
Med.	10	53.5	100	5.35
Hi	40	53.5	100	20.4

J. RESULTS

1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

2. LOW POINT VALIDATION

Results for this evaluation appears in Table 2.

3. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

   Results for this evaluation appears in Table 3.

3. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 4A-D.

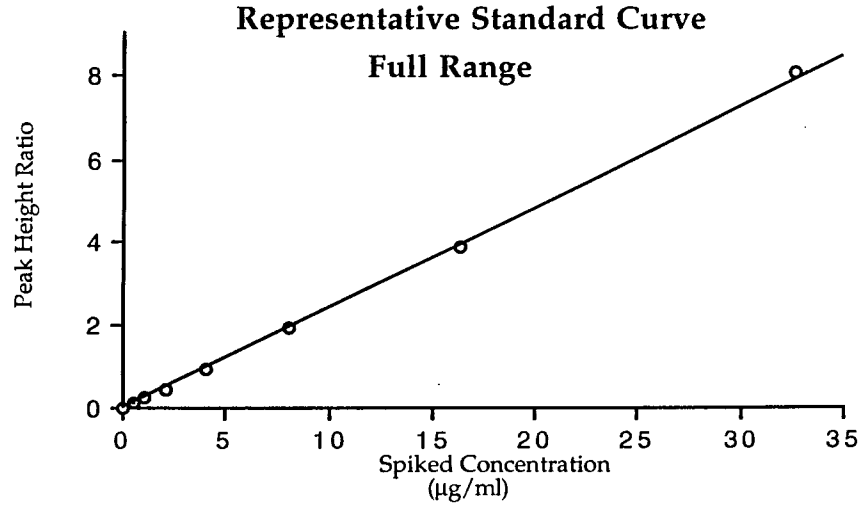
4. RECOVERY

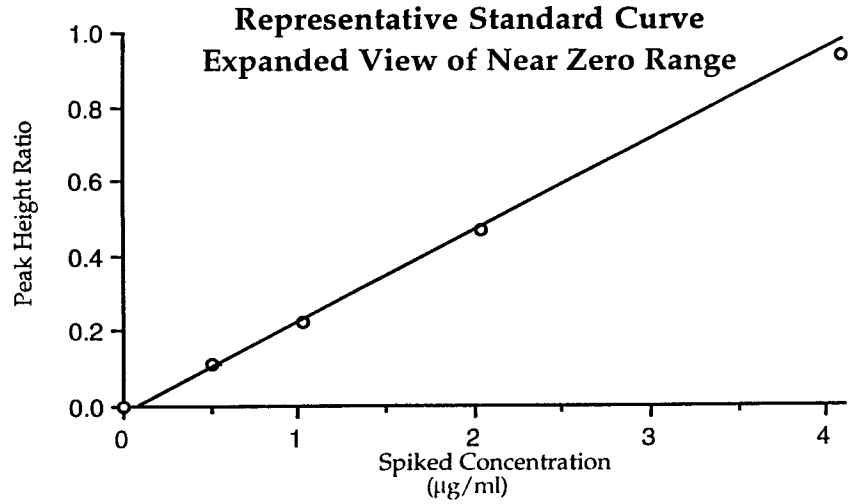
   Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR HALOFANTRINE (FREE BASE) RAT PERFUSATE PRECIPITATION ASSAY, STUDY REPORT 17B, SUPPLEMENT NO. I

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (µg/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (µg/ml)
0	0	0	-
52.0	0.520	0.093	0.568
104	1.04	0.172	0.992
208	2.08	0.366	2.03
416	4.16	0.751	4.10
832	8.32	1.526	8.26
1664	16.64	3.064	16.5
3328	33.28	6.238	33.6

Regression equation:***
$y = 0.186x - 0.0127$, $r^2 = 0.9998$



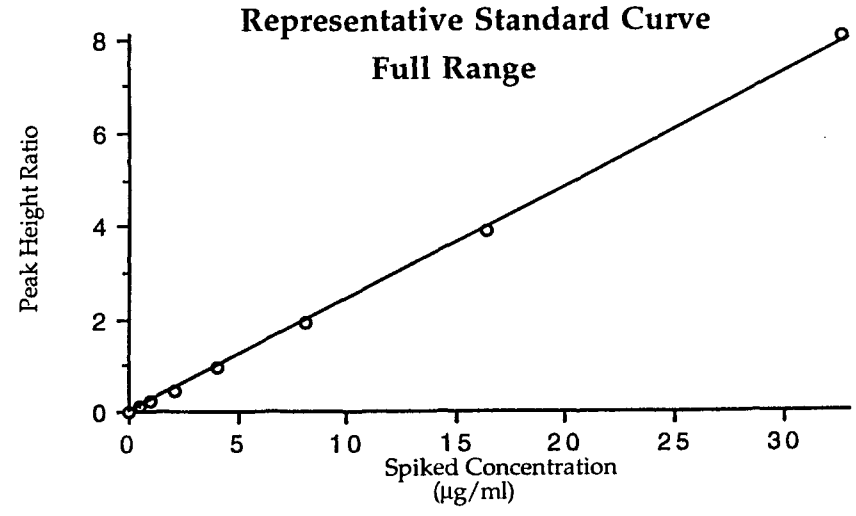


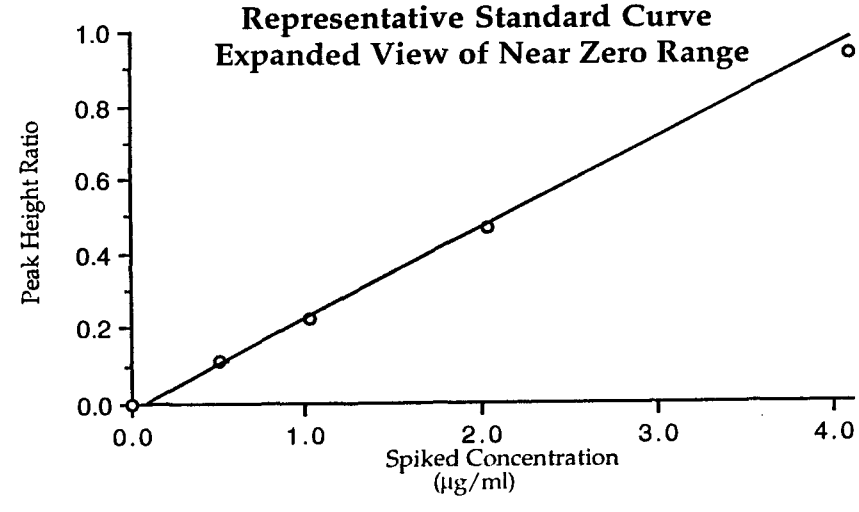
* Into 100 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR WR 178,460 (FREE BASE) RAT PERFUSATE PRECIPITATION ASSAY, STUDY REPORT 17B, SUPPLEMENT NO. I

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (µg/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (µg/ml)
0	0	0	-
51.0	0.510	0.115	0.553
102	1.02	0.224	1.00
204	2.04	0.466	2.00
408	4.08	0.939	3.95
816	8.16	1.920	7.99
1632	16.32	3.896	16.1
3264	32.64	8.028	33.2

Regression equation:*** $y = 0.243x - 0.0192$, $r^2 = 0.9996$





* Into 100 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 2: LOW POINT VALIDATION OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) RAT PERFUSATE PRECIPITATION ASSAY

Spiked Concentration	HALOFANTRINE (free base) (0.520 µg/ml)		WR 178,460 (free base) (0.510 µg/ml)	
	Measured Concentrations (µg/ml)			
	Interday	Intraday	Interday	Intraday
	0.568	0.482	0.553	0.543
	0.545	0.459	0.554	0.561
	0.546	0.523	0.558	0.600
		0.593		0.517
		0.476		0.504
		0.476		0.622
Mean	0.553	0.502	0.555	0.558
S. D.	0.013	0.050	0.003	0.046
Percent C.V.	2.35	9.90	0.48	8.29
Percent R.E.	6.35	-3.56	8.82	9.38

TABLE 3: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE AND WR 178,460 AS FREE BASES RAT PERFUSATE PRECIPITATE ASSAY, STUDY REPORT 17B, SUPPLEMENT I

Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
Halofantrine				
4/7/94	1(intraday)	0.18495382	-0.00766061	0.999565216
4/8/94	2(interday 1)	0.186197073	-0.012688465	0.999762579
4/8/94	3(interday 2)	0.186384551	-0.018650289	0.99982947
4/8/94	4(Interday 3)	0.186839457	-0.016978611	0.999406029
WR 178460				
4/7/94	1A(intraday)	0.242112571	-0.021091236	0.99937132
4/8/94	2A(interday 1)	0.242656365	-0.019223641	0.999551985
4/8/94	3A(interday 2)	0.242181938	-0.026159153	0.999610049
4/8/94	4A(Interday 3)	0.244046863	-0.023255414	0.998919693

Halofantrine Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml) 0.52	1.04	2.08	4.16	8.32	16.64	33.28
	Back Calculated Concentration (µg/ml)						
1	0.571	1.01	2.03	4.07	8.09	16.5	33.8
2	0.568	0.992	2.03	4.10	8.26	16.5	33.6
3	0.545	1.02	2.02	4.12	8.29	16.9	33.1
4	0.546	1.05	2.04	4.05	7.93	17.0	33.5
Mean	0.558	1.02	2.03	4.09	8.14	16.7	33.5
S.D.	0.014	0.024	0.008	0.031	0.167	0.263	0.294
Percent C.V.	2.50	2.38	0.402	0.761	2.05	1.57	0.879
Percent R.E.	7.21	-2.12	-2.40	-1.80	-2.13	0.511	0.661

WR 178460 Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml) 0.510	1.02	2.04	4.08	8.16	16.32	32.64
	Back Calculated Concentration (µg/ml)						
1A	0.554	1.01	2.01	3.94	7.86	16.2	33.2
2A	0.553	1.00	2.00	3.95	7.99	16.1	33.2
3A	0.554	1.00	1.96	3.96	7.95	16.5	32.8
4A	0.558	1.03	1.96	3.91	7.68	16.6	33.1
Mean	0.555	1.01	1.98	3.94	7.87	16.4	33.1
S.D.	0.002	0.014	0.026	0.022	0.138	0.238	0.189
Percent C.V.	0.400	1.40	1.33	0.548	1.75	1.46	0.572
Percent R.E.	8.77	-0.98	-2.82	-3.43	-3.55	0.184	1.33

TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT PERFUSION PRECIPITATION ASSAY

Interday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/mL) 1.18	5.90	23.6
		Measured Concentrations (µg/mL)		
2	1	1.16	5.49	23.1
	2	1.20	5.42	22.9
3	1	1.18	5.92	23.8
	2	1.18	5.91	23.9
4	1	1.19	5.46	22.4
	2	1.16	5.53	23.1
Mean		1.18	5.62	23.2
S.D.		0.016	0.23	0.566
Percent C.V.		1.36	4.09	2.44
Percent R.E.		-0.141	-4.72	-1.69

TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT PERFUSION PRECIPITATION ASSAY

Intraday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/mL) 1.18	5.90	23.6
		Measured Concentrations (µg/mL)		
1	1	1.10	5.60	23.2
	2	1.11	5.83	23.0
	3	1.09	5.79	23.3
	4	1.13	5.49	22.7
	5	1.17	5.52	22.3
	6	1.19	5.30	23.0
Mean		1.13	5.59	22.9
S.D.		0.040	0.198	0.37
Percent C.V.		3.55	3.55	1.60
Percent R.E.		-4.10	-5.28	-2.90

TABLE 4C: PRECISION OF WR 178,460 FREE BASE RAT PERFUSION PRECIPITATION ASSAY

Interday Precision WR 178460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/mL) 1.07	5.35	20.4
		Measured Concentrations (µg/mL)		
2A	1	1.02	5.01	21.1
	2	1.06	4.99	21.2
3A	1	1.12	5.33	21.9
	2	1.07	5.31	21.8
4A	1	1.06	4.92	20.5
	2	1.10	4.96	21.4
Mean		1.07	5.09	21.3
S.D.		0.0349	0.183	0.512
Percent C.V.		3.25	3.61	2.40
Percent R.E.		0.156	-4.92	4.49

TABLE 4D: PRECISION OF WR 178,460 FREE BASE RAT PERFUSION PRECIPITATION ASSAY

Intraday Precision WR 178460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/mL) 1.07	5.35	20.4
		Measured Concentrations (µg/mL)		
1A	1	1.09	5.08	21.5
	2	1.07	5.34	21.5
	3	1.02	5.22	21.4
	4	1.06	4.96	21.2
	5	1.08	5.02	20.4
	6	1.07	4.87	20.9
Mean		1.07	5.08	21.2
S.D.		0.024	0.173	0.43
Percent C.V.		2.28	3.40	2.04
Percent R.E.		-0.467	-5.02	3.68

TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT PERFUSION BY PRECIPITATION

SAMPLE ID	SPIKED CONCENTRATION Range	(µg/ml)	PEAK HEIGHT RATIO REFERENCE	PERFUSATE	MEAN PERCENT RECOVERY
Halofantrine					
1	Low	1.18	0.100	0.101	101
2			0.104	0.122	
3			0.120	0.105	
Mean (± SD)			0.108 ± 0.011	0.109 ± 0.011	
1	Medium	5.90	0.611	0.596	100
2			0.607	0.609	
3			0.575	0.590	
Mean (± SD)			0.598 ± 0.020	0.598 ± 0.010	
1	High	23.6	2.457	2.255	96.0
2			2.354	2.363	
3			2.413	2.314	
Mean (± SD)			2.408 ± 0.052	2.311 ± 0.054	
AVERAGE RECOVERY =					99.1
WR 178,460					
1	Low	1.07	0.122	0.124	106
2			0.119	0.140	
3			0.138	0.138	
Mean (± SD)			0.126 ± 0.010	0.134 ± 0.009	
1	Medium	5.35	0.696	0.698	101
2			0.684	0.701	
3			0.669	0.678	
Mean (± SD)			0.683 ± 0.014	0.692 ± 0.013	
1	High	20.4	2.843	2.575	93.6
2			2.773	2.657	
3			2.800	2.649	
Mean (± SD)			2.805 ± 0.035	2.627 ± 0.045	
OVERALL AVERAGE RECOVERY =					100

LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460 (AS FREE BASES) IN RAT PERFUSATE EXTRACTION ASSAY, STUDY REPORT 17, SUPPLEMENT II

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.

2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.

3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.

4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.

2. All chemicals are reagent grade.

3. Halofantrine hydrochloride (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BB43807, expiration date not available.

4. WR 178,460 (hydrochloride) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK21070, expiration date not available.

5. WR 122,455 (internal standard) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. AX26839, expiration date not available.

6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).

7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).

8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).

9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

C. ASSAY CONDITIONS

1. DETECTOR

Settings

Wavelength: excitation - 300 nm, emission - 375 nm
Sensitivity: high
Range: 4
Response: medium

Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

2. COLUMN

Axxiom Silica, 5 µm particle size, 4.6 x 250 mm
(Richard Scientific, Novato, CA).

3. SOLVENT SYSTEM

Combine and mix H_2O (800 m L) + $(NH_4)_2HPO_4$ (20 mL of 1 M $(NH_4)_2HPO_4$) + CH_3OH (3200 L).

4. FLOW RATE

1.0 ml/min

5. STOCK SOLUTIONS - Solutions were stored in the 20°C freezer (refrigerator) and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (standard solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

A. Stock Solutions

i. HALOFANTRINE - (Halofantrine Hydrochloride, WRAIR, Washington, D.C.), bottle number BB 43807.

Prep date: 3/1/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.60	0.942	50.6	methanol	104
Precision	6.24	0.942	50.0	methanol	118

*= Molecular weights of halofantrine free base/halofantrine hydrochloride

ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride, free base concentrations).

Prep date: 3/1/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.60	0.924	50.6	methanol	102
Precision	6.03	0.924	52.0	methanol	107

*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

iii. WR 122,455 (Internal Standard) - (WRAIR, Washington, D.C.).

Prep date: 12/14/93

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	5.20	1	25	methanol	208

B. Working Solutions

A. Halofantrine and WR 178,460 Mixed Working Solutions

1. High Concentration Working Solution: Combine 8.00 ml each of halofantrine and WR 178,460 (as free bases) stock solutions.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	8.00	100	methanol	8.32
Precision (Halofantrine)	118	8.00	100	methanol	9.44
Standard Curve (WR 178,460)	102	8.00	100	methanol	8.16
Precision (WR 178,460)	107	8.00	100	methanol	8.56

2. Low Concentration Working Solution: Combine 1.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 100 ml.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	1.00	100	methanol	1.04
Precision (Halofantrine)	118	1.00	100	methanol	1.18

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (WR 178,460)	102	1.00	100	methanol	1.02
Precision (WR 178,460)	107	1.00	100	methanol	1.07

B. WR 122,455 - Internal standard.

Prep date: 3/4/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	208	0.5	100	methanol	1.04

7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.

   a. Halofantrine - 7.4 min
   b. WR 178,460 - 11 min
   c. WR 122,455 (Internal Standard) - 13 min

8. BLANK RAT PERFUSATE

   Supplied by WRAIR.

9. INJECTION VOLUME

   50 - 100 µl

10. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of $1/y_i$.

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat perfusate were based on the interday and intraday low point validation results (Table 3) and on standard curve calibrator results (Tables 1 and 2).)

    10.4 ng/ml halofantrine (free base).
    10.2 ng/ml WR 178,460 (free base).

12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

    Perfusate samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. The drug is spiked with a Hamilton syringe.

13. WISP OPERATING TEMPERATURE

    Room temperature.

D. SAMPLE STORAGE

All samples are to be kept frozen at -20°C before analysis and thawed at room temperature for preparation (within 30 min.) and analysis.

E. SAMPLE PREPARATION

1. Pipet 100 µl rat perfusate samples into 16 X 125 silanized tubes on ice. Add 100 µl water.
2. Add 20 µl of 1.04 µg/ml WR 122,455 internal standard solution. Vortex for 30s.
3. Add 50 µl 0.1N NaOH. Vortex 30 s.
4. Add 2.0 ml methyl *t*-butyl ether. Vortex 1 min., twice. Centrifuge for 10 min. at 3000*g*.
5. Freeze aqueous layer in dry ice/methanol bath and pour organic layer into a 13 x 100 silanized tube.
6. Repeat steps 5 and 6.
7. Evaporate to dryness. Reconstitute in 4:1 methanol/water (v/v) with a final 0.001% HCl concentration.
8. Transfer supernatant to silanized inserts and inject onto column.

F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 100 µl perfusate samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

Generation of Halofantrine Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (ng/ml)
00	0	0	0	0
0	0	0	0	0
1	1	1.04	1.04	10.4
2	2	1.04	2.08	20.8
3	4	1.04	4.16	41.6
4	8	1.04	8.32	83.2
5	2	8.32	16.64	166.4
6	4	8.32	33.28	333
7	8	8.32	66.56	666
8	16	8.32	133.12	1331

Generation of WR 178,460 Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (ng/ml)
00	0	0	0	0
0	0	0	0	0
1	1	1.02	1.02	10.2
2	2	1.02	2.04	20.4
3	4	1.02	4.08	40.8
4	8	1.02	8.16	81.6
5	2	8.16	16.32	163
6	4	8.16	32.64	326
7	8	8.16	65.28	653
8	16	8.16	130.56	1306

G. QUALITY CONTROL

1. Content and frequency of blanks

   No special blank was used except for the standard curve blank.

2. Pipette Calibration

   See ASOP 2C-1.1.

3. Balance Calibration

   See ASOP 2C-2.1.

H. RECOVERY

Assay recovery was assessed at four different halofantrine and WR 178,460 (as free bases) concentrations (equal to precision sample nominal concentrations) by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in perfusate (100 µl) samples. The perfusate samples were generated by spiking on ice 100 µl of blank rat perfusate to which water (100 µl) has been added with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). Each perfusate sample was prepared as described in "Sample Preparation" above, except the internal standard was added to the transferred organic layer prior to evaporation. The reference samples (100 µl rat perfusate) were generated by spiking methyl *t*-butyl ether extracts of rat perfusate (obtained as described in "Sample Preparation" above) with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). These reference samples were then prepared by completion of the sample preparation procedure as described (addition of IS, evaporation and reconstitution).

I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 100 µl perfusate specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Halofantrine Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (ng/ml)
XL	2	1.18	100	23.6
Low	6	1.18	100	70.8
Med.	3	9.44	100	283.2
Hi	10	9.44	100	944

Generation of WR 178,460 Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (ng/ml)
XL	2	1.07	100	21.4
Low	6	1.07	100	64.2
Med.	3	8.56	100	257
Hi	10	8.56	100	856

J. RESULTS

1. STANDARD CURVE

   Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

2. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

   Results for this evaluation appears in Table 2.

3. LOW POINT VALIDATION

   Results for this evaluation appears in Table 3.

4. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 4A-D.

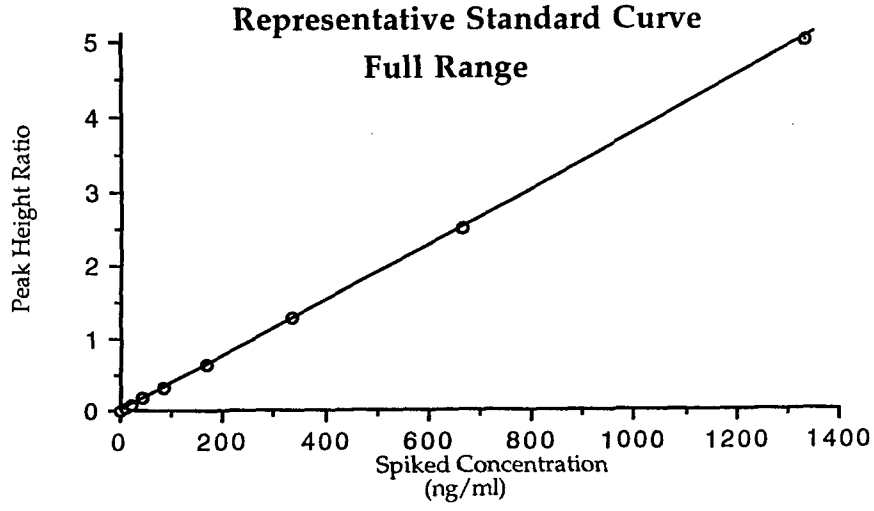
5. RECOVERY

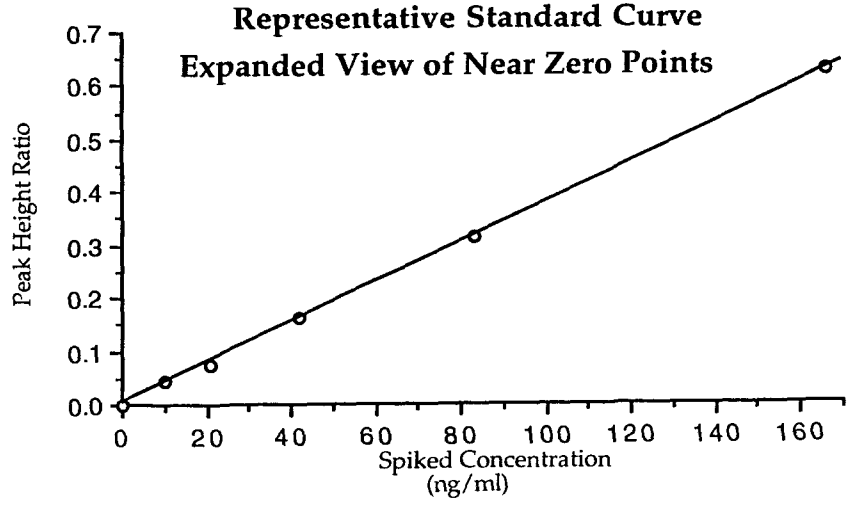
   Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR HALOFANTRINE (FREE BASE) RAT PERFUSATE EXTRACTION ASSAY, STUDY REPORT 17, SUPPLEMENT NO. 2

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
1.04	10.4	0.046	11.5
2.08	20.8	0.073	18.7
4.16	41.6	0.163	42.6
8.32	83.2	0.312	82.3
16.64	166.4	0.625	166
33.28	333	1.269	337
66.56	666	2.496	663
133.12	1331	5.009	1330

Regression equation:***
$y = 0.00376x + 0.00269$, $r^2 = 0.9998$



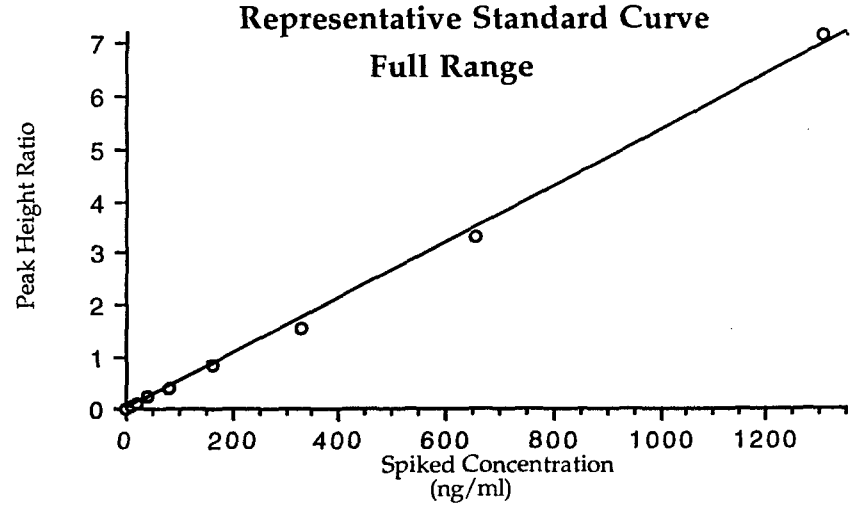


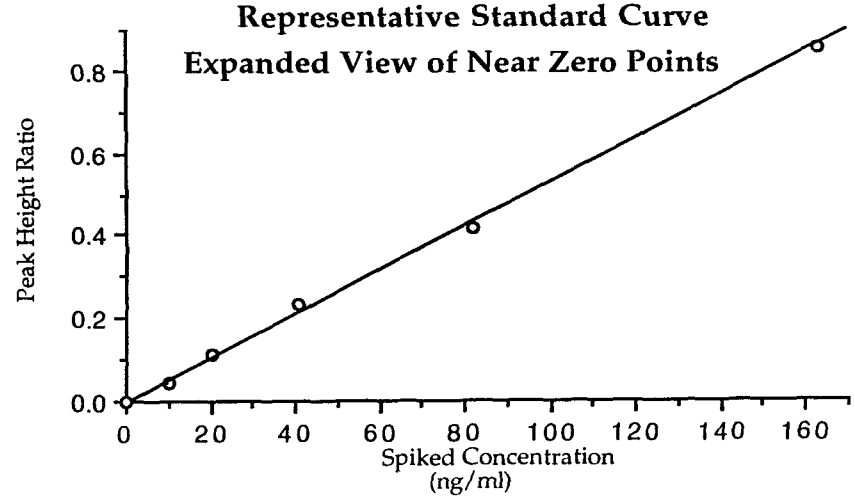
* Into 100 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR WR 178,460 (FREE BASE) RAT PERFUSATE EXTRACTION ASSAY, STUDY REPORT 17, SUPPLEMENT NO. 2

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
1.02	10.2	0.042	9.22
2.04	20.4	0.116	23.2
4.08	40.8	0.235	45.6
8.16	81.6	0.414	79.4
16.32	163	0.859	163
32.64	326	1.583	300
65.28	653	3.346	632
130.56	1306	7.174	1350

Regression equation:*** $y = 0.00530x - 0.00691$, $r^2 = 0.9975$





* Into 100 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 2: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE AND WR 178,460 AS FREE BASES RAT PERFUSATE EXTRACTION ASSAY, STUDY REPORT 17B, SUPPLEMENT II

Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
Halofantrine				
5/27/94	1(interday 1)	0.003758804	0.002694172	0.999815534
6/2/96	2(intraday)	0.003635292	-0.008268307	0.997896349
6/2/96	3(interday 2)	0.003507602	0.003106297	0.999308123
6/18/94	4(interday 3)	0.004056372	0.007003992	0.999050706
WR 178460				
5/27/94	1A(interday 1)	0.005303248	-0.006905876	0.997504309
6/2/96	2A(intraday)	0.005186956	0.008207105	0.998102803
6/2/96	3A(interday 2)	0.005065837	0.019508885	0.999760483
6/18/94	4A(interday 3)	0.00529495	0.001261078	0.999771288

Halofantrine Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (ng/ml)							
	10.4	20.8	41.6	83.2	166.4	333	666	1331
	Back Calculated Concentration (ng/ml)							
1	11.5	18.7	42.6	82.3	166	337	663	1330
2	11.7	19.1	38.2	83.2	176	341	671	1310
3	10.4	20.7	45.4	75.9	163	352	659	1330
4	12.7	16.9	39.4	83.7	176	361	652	1320
Mean	11.6	18.9	41.4	81.3	170	348	661	1323
S.D.	0.943	1.56	3.25	3.63	6.75	10.9	7.93	9.57
Percent C.V.	8.15	8.28	7.85	4.47	3.97	3.13	1.20	0.72
Percent R.E.	11.3	-9.38	-0.48	-2.31	2.31	4.43	-0.71	-0.64

WR 178460 Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (ng/ml)							
	10.2	20.4	40.8	81.6	163	326	653	1306
	Back Calculated Concentration (ng/ml)							
1A	9.22	23.2	45.6	79.4	163	300	632	1350
2A	11.5	20.2	39.2	80	162	318	654	1320
3A	10.3	21.3	38.5	81.7	163	331	641	1310
4A	11.7	19.2	42.2	72.6	176	318	628	1340
Mean	10.7	21.0	41.4	78.4	166	317	639	1330
S.D.	1.15	1.71	3.24	4.00	6.68	12.7	11.5	18.3
Percent C.V.	10.8	8.17	7.84	5.11	4.03	4.02	1.80	1.37
Percent R.E.	4.71	2.82	1.41	-3.89	1.84	-2.84	-2.18	1.84

TABLE 3: LOW POINT VALIDATION OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) RAT PERFUSATE EXTRACTION ASSAY

Spiked Concentration	HALOFANTRINE (free base) (10.4 ng/ml)		WR 178,460 (free base) (10.2 ng/ml)	
	Measured Concentrations (ng/ml)			
	Interday	Intraday	Interday	Intraday
	11.7	12.5	9.28	10.1
	11.7	11.0	11.5	9.52
	10.4	10.4	10.3	10.4
		11.7		11.6
		11.2		10.8
		11.7		11.6
Mean	11.3	11.4	10.4	10.7
Standard Deviation	0.751	0.719	1.11	0.833
Percent C.V.	6.66	6.30	10.7	7.80
Percent R.E.	8.33	9.78	1.57	4.61

TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT PERFUSATE EXTRACTION ASSAY

Interday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (ng/ml) 23.6	70.8	283.2	944
		Measured Concentrations (ng/ml)			
1	1	21.6	65.3	278	845
	2	18.7	67.1	261	851
3	1	21.4	70.4	280	935
	2	23.3	61.3	286	940
4	1	21.4	66.3	262	888
	2	bc	72.7	298	912
Mean		21.3	67.2	278	895
S.D.		1.65	3.99	14.2	41
Percent C.V.		7.75	5.94	5.13	4.58
Percent R.E.		-9.83	-5.11	-2.01	-5.17

TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT PERFUSATE EXTRACTION ASSAY

Intraday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (ng/ml) 23.6	70.8	283.2	944
		Measured Concentrations (ng/ml)			
2	1	24.0	73.0	255	919
	2	21.3	67.5	272	903
	3	24.3	72.1	270	917
	4	25.7	70.2	265	909
	5	21.8	72.1	278	912
	6	24.3	70.5	262	882
Mean		23.6	70.9	267	907
S.D.		1.68	1.97	8.10	13.5
Percent C.V.		7.12	2.79	3.03	1.49
Percent R.E.		-0.14	0.14	-5.72	-3.92

TABLE 4C: INTERDAY PRECISION OF WR 178,460 FREE BASE RAT PERFUSATE EXTRACTION ASSAY

Interday Precision WR 178,460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (ng/ml) 21.4	64.2	257	856
		Measured Concentrations (ng/ml)			
1A	1	24.1	62	257	780
	2	22.2	60.3	237	776
3A	1	20.4	64.3	257	869
	2	22.4	66	264	851
4A	1	19.8	59.4	245	807
	2	bc	60.6	265	842
Mean		21.8	62.1	254	821
S.D.		1.72	2.56	11	38.9
Percent C.V.		7.88	4.13	4.34	4.73
Percent R.E.		1.78	-3.27	-1.1	-4.11

TABLE 4D: INTRADAY PRECISION OF WR 178,460 FREE BASE RAT PERFUSATE EXTRACTION ASSAY

Intraday Precision WR 178,460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (ng/ml) 21.4	64.2	257	856
		Measured Concentrations (ng/ml)			
2A	1	21.7	64.0	234	846
	2	22.3	69.6	252	834
	3	23.7	62.2	244	851
	4	24.1	53.7	243	843
	5	20.6	59.3	252	840
	6	21.6	57.8	233	844
Mean		22.3	61.1	243	843
S.D.		1.34	5.49	8.29	5.73
Percent C.V.		5.99	8.99	3.41	0.68
Percent R.E.		4.36	-4.83	-5.45	-1.52

TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT PERFUSATE BY EXTRACTION

SAMPLE ID	SPIKED CONCENTRATION Range	(µg/ml)	PEAK HEIGHT RATIO REFERENCE	PERFUSATE	MEAN PERCENT RECOVERY
Halofantrine					
1	Extra Low	23.6	0.115	0.100	84.2
2			0.119	0.096	
3			0.114	0.097	
Mean (± SD)			0.116 ±0.003	0.098 ±0.002	
1	Low	70.8	0.294	0.327	95.5
2			0.287	0.298	
3			0.358	0.272	
Mean (± SD)			0.313 ±0.039	0.299 ±0.028	
1	Medium	283.2	1.143	1.085	91.9
2			1.164	1.055	
3			1.199	1.081	
Mean (± SD)			1.169 ±0.028	1.074 ±0.016	
1	High	944	3.426	3.624	91.7
2			3.811	3.153	
3			3.756	3.306	
Mean (± SD)			3.664 ±0.208	3.361 ±0.240	
AVERAGE MEAN RECOVERY = 90.8					
WR 178,460					
1	Extra Low	21.4	0.116	0.111	91.3
2			0.132	0.108	
3			0.118	0.115	
Mean (± SD)			0.122 ±0.009	0.111 ±0.004	
1	Low	64.2	0.334	0.317	91.8
2			0.371	0.317	
3			0.354	0.338	
Mean (± SD)			0.353 ±0.019	0.324 ±0.012	
1	Medium	257	1.369	1.314	93.2
2			1.341	1.236	
3			1.415	1.295	
Mean (± SD)			1.375 ±0.037	1.282 ±0.041	
1	High	856	4.066	4.372	93.3
2			4.488	3.699	
3			4.486	4.099	
Mean (± SD)			4.347 ±0.243	4.057 ±0.338	
AVERAGE MEAN RECOVERY = 92.4					

LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460 AS FREE BASES IN RAT BILE PRECIPITATION ASSAY, STUDY REPORT 17, SUPPLEMENT III

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Halofantrine hydrochloride, bottle no. BB 43807 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
4. WR 178,460 (hydrochloride), bottle no. BK 21070, (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
5. WR 122,455 (internal standard), bottle no. AX 26839 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

C. ASSAY CONDITIONS

1. DETECTOR

Settings

Wavelength: excitation - 300 nm, emission - 375 nm
Sensitivity: high
Range: 4
Response: medium

Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

2. COLUMN

Axxiom Silica, 5 µm particle size, 4.6 x 250 mm
(Richard Scientific, Novato, CA).

3. SOLVENT SYSTEM

Combine and mix H_2O (800 ml) + $(NH_4)_2HPO_4$ (20 ml of 1 M $(NH_4)_2HPO_4$) + CH_3OH (3200 ml).

4. FLOW RATE

1.0 ml/min.

5. STOCK SOLUTIONS - Solutions were stored in the -20°C freezer (refrigerator) and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

A. Stock Solutions

i. HALOFANTRINE - (Halofantrine Hydrochloride).

Prep date: 3/1/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.60	0.942	50.6	methanol	104
Precision	6.24	0.942	50.0	methanol	118

*= Molecular weights of halofantrine free base/halofantrine hydrochloride

ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride).

Prep date: 3/1/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.60	0.924	50.6	methanol	102
Precision	6.03	0.924	52.0	methanol	107

*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

iii. WR 122,455 (Internal Standard).

Prep date: 3/4/94

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	5.20	1	25	methanol	208

B. Working Solutions

i. Halofantrine and WR 178,460 Mixed Working Solutions

a. HIGH CONCENTRATION WORKING SOLUTION - Combine 10.0 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	10.0	20.0	methanol	52.0
Standard Curve (WR 178,460)	102	10.0	20.0	methanol	51.0
Precision (Halofantrine)	118	10.0	20.0	methanol	59.0
Precision (WR 178,460)	107	10.0	20.0	methanol	53.5

b. LOW CONCENTRATION WORKING SOLUTION - Combine 1.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 10 ml.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	1.00	10.0	methanol	10.4
Standard Curve (WR 178,460)	102	1.00	10.0	methanol	10.2

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Precision (Halofantrine)	118	1.00	10.0	methanol	11.8
Precision (WR 178,460)	107	1.00	10.0	methanol	10.7

ii. WR 122,455 - Internal standard.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (part)	QS Volume (part)	Solvent	Conc. (µg/ml)
Internal std.	208	0.5	10.5	methanol	9.90

7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.
   a. Halofantrine - 7.2 min.
   b. WR 178,460 - 10.7 min.
   c. WR 122,455 (Internal Standard) - 12.7 min.

8. BLANK RAT BILE
   Supplied by WRAIR.

9. INJECTION VOLUME
   20 µl

10. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of $1/y_i$.

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat bile were based on the interday and intraday low point validation results (Table 3).)

    0.416 µg/ml halofantrine (free base).

    0.408 µg/ml WR 178,460 (free base).

12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

    Bile samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. Drugs are spiked with Hamilton syringes.

13. WISP OPERATING TEMPERATURE
    Room temperature.

D. SAMPLE STORAGE

All samples are to be kept frozen at -70°C before analysis and thawed at room temperature for preparation (within 30 min.) and analysis.

E. SAMPLE PREPARATION

1. Pipet 25 µl rat bile samples into 13 X 100 silanized tubes.
2. Add 10 µl of 9.90 µg/ml WR 122,455 internal standard solution. Vortex for 30s.
3. Add 0.2 ml CH_3CN. Vortex 1 min.
4. Centrifuge at 3000 *g* for 10 min.
5. Transfer supernatant to silanized inserts and inject onto column.

F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 25 µl bile samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

Generation of Halofantrine Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (µg)	Standard Curve Sample Nominal Concentration (µg/ml)
00	0	10.4	0	0
0	0	10.4	0	0
1	1	10.4	10.4	0.416
2	2	10.4	20.8	0.832
3	4	10.4	41.6	1.664
4	8	10.4	83.2	3.328
5	4	52.0	208	8.320
6	8	52.0	416	16.64
7	16	52.0	832	33.28
8	32	52.0	1664	66.56

Generation of WR 178,460 Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (µg)	Standard Curve Sample Nominal Concentration (µg/ml)
00	0	10.2	0	0
0	0	10.2	0	0
1	1	10.2	10.2	0.408
2	2	10.2	20.4	0.816
3	4	10.2	40.8	1.632
4	8	51.0	81.6	3.264
5	4	51.0	204	8.16
6	8	51.0	408	16.32
7	16	51.0	816	32.64
8	32	51.0	1632	65.28

G. QUALITY CONTROL

1. Content and frequency of blanks

   No special blank was used except for the standard curve blank.

2. Pipette Calibration

   See ASOP 2C-1.1.

3. Balance Calibration

   See ASOP 2C-2.1.

H. RECOVERY

Assay recovery was assessed at four different concentrations by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in bile samples. Bile (25 µl) and reference (25 µl water) samples were spiked with corresponding amounts of halofantrine and WR 178,460 (as free bases) prior to addition of acetonitrile precipitant. Each bile and reference sample was prepared as described in "Sample Preparation" (Section E), except the internal standard was added to 150 µl of the transferred supernatant (after step 5, not in step 3).

I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 25 µl bile specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Halofantrine Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (µg/ml)
XL	2	11.8	25	0.944
Low	10	11.8	25	4.72
Med.	6	59.0	25	14.16
Hi	12	59.0	25	28.32

Generation of WR 178,460 Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (µg/ml)
XL	2	10.7	25	0.856
Low	10	10.7	25	4.28
Med.	6	53.5	25	12.84
Hi	12	53.5	25	25.68

J. RESULTS

1. STANDARD CURVE

   Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

2. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

   Results for this evaluation appears in Table 2.

3. LOW POINT VALIDATION

   Results for this evaluation appears in Table 3.

4. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 4A-D.

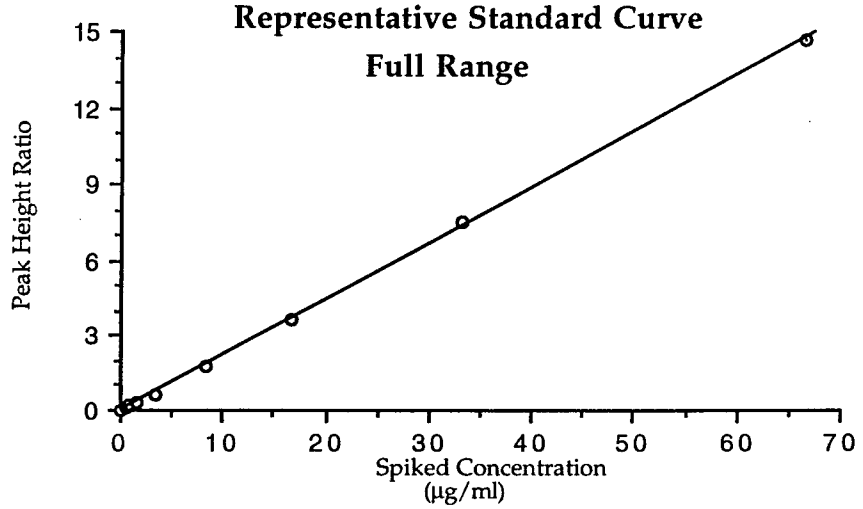
5. RECOVERY

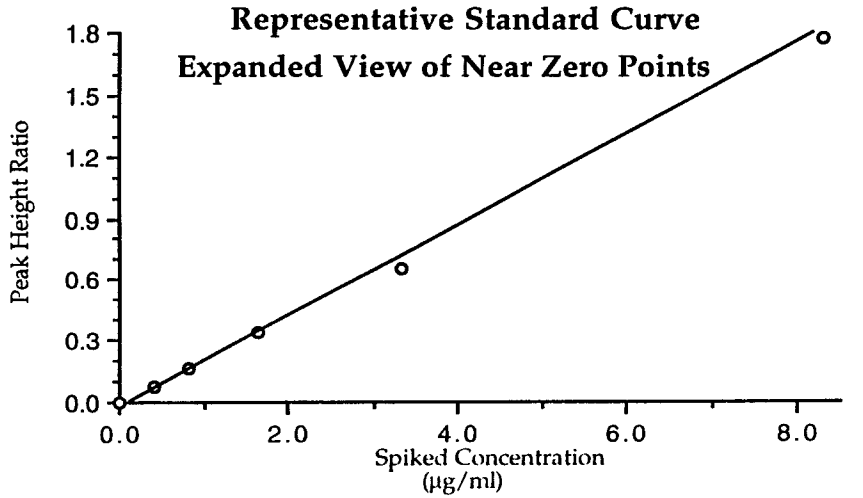
   Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR HALOFANTRINE (FREE BASE) RAT BILE PRECIPITATION ASSAY, STUDY REPORT 17, SUPPLEMENT NO. III

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (µg/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (µg/ml)
0	0	-	-
10.4	0.416	0.080	0.460
20.8	0.832	0.166	0.848
41.6	1.664	0.336	1.62
83.2	3.328	0.652	3.04
208	8.32	1.770	8.10
416	16.64	3.632	16.5
832	33.28	7.509	34.0
1664	66.56	14.691	66.5

Regression equation:***
$y = 0.221x - 0.0217$, $r^2 = 0.9995$



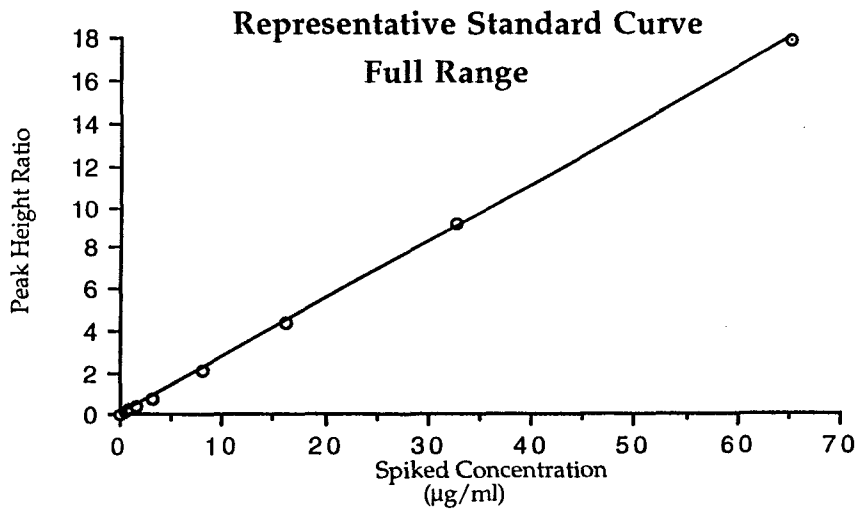


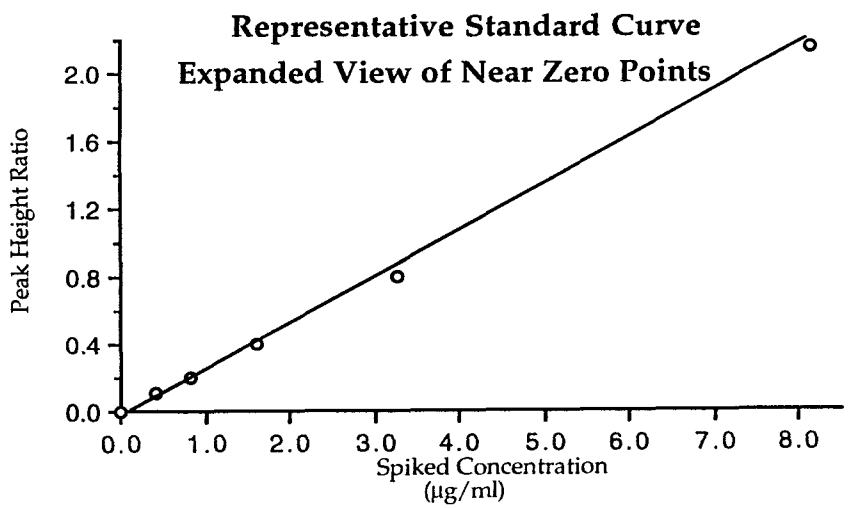
* Into 25 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR WR 178,460 (FREE BASE) RAT BILE PRECIPITATION ASSAY, STUDY REPORT 17, SUPPLEMENT NO. III

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (µg/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (µg/ml)
0	0	-	-
10.2	0.408	0.104	0.466
20.4	0.816	0.201	0.82
40.8	1.632	0.401	1.55
81.6	3.264	0.805	3.02
204	8.16	2.150	7.92
408	16.32	4.416	16.2
816	32.64	9.155	33.4
1632	65.28	17.879	65.2

Regression equation:***
$y = 0.243x - 0.0192$, $r^2 = 0.9996$





* Into 25 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 2: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE AND WR 178,460 AS FREE BASES RAT PERFUSATE EXTRACTION ASSAY, STUDY REPORT 17B, SUPPLEMENT II

Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
Halofantrine				
3/23/94	1(interday 1)	0.21963467	-0.0108649	0.99934181
3/23/96	2(intraday)	0.22128084	-0.0216939	0.99950683
3/24/96	3(interday 2)	0.2229092	-0.0150255	0.99911694
3/26/94	4(interday 3)	0.22293755	-0.0162028	0.99973786
WR 178460				
3/23/94	1A(interday 1)	0.2606906	-0.0236581	0.99780183
3/23/96	2A(intraday)	0.27455641	-0.0239993	0.99946485
3/24/96	3A(interday 2)	0.26407302	-0.0230908	0.99855242
3/26/94	4A(interday 3)	0.2391856	-0.0166697	0.99554945

Halofantrine Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml)							
	0.416	0.832	1.664	3.328	8.32	16.64	33.28	66.56
	Back Calculated Concentration (µg/ml)							
1	0.459	0.864	1.63	3.17	7.77	16.3	33.2	67.7
2	0.460	0.848	1.62	3.04	8.10	16.5	34.0	66.5
3	0.458	0.897	1.62	2.95	8.08	16.2	34.2	66.8
4	0.472	0.817	1.61	3.12	8.19	16.6	33.2	67.1
Mean	0.462	0.857	1.62	3.07	8.04	16.4	33.7	67.0
S.D.	0.0066	0.033	0.0082	0.096	0.183	0.183	0.526	0.512
Percent C.V.	1.42	3.89	0.504	3.14	2.28	1.11	1.56	0.764
Percent R.E.	11.1	2.94	-2.64	-7.75	-3.43	-1.44	1.11	0.699

WR 178460 Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml)							
	0.408	0.816	1.632	3.264	8.16	16.32	32.64	65.28
	Back Calculated Concentration (µg/ml)							
1A	0.474	0.839	1.56	3.26	7.26	15.5	32.3	67.6
2A	0.466	0.820	1.55	3.02	7.92	16.2	33.4	65.2
3A	0.500	0.856	1.49	2.91	7.82	15.6	33.7	65.7
4A	0.475	0.801	1.70	2.93	7.92	16.2	29.9	69.1
Mean	0.479	0.829	1.58	3.03	7.73	15.9	32.3	66.9
S.D.	0.015	0.024	0.089	0.161	0.317	0.377	1.73	1.79
Percent C.V.	3.08	2.87	5.64	5.30	4.10	2.38	5.34	2.68
Percent R.E.	17.3	1.59	-3.49	-7.17	-5.27	-2.73	-0.965	2.48

TABLE 3: LOW POINT VALIDATION OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) RAT BILE PRECIPITATION ASSAY

Spiked Concentration	HALOFANTRINE (free base) (0.416 µg/ml)		WR 178,460 (free base) (0.408 µg/ml)	
	Measured Concentrations (µg/ml)			
	Interday	Intraday	Interday	Intraday
	0.459	0.480	0.474	0.524
	0.458	0.418	0.500	0.465
	0.472	0.523	0.475	0.458
		0.451		0.423
		0.537		0.462
		0.451		0.477
Mean	0.463	0.477	0.483	0.468
Standard Deviation	0.008	0.046	0.015	0.033
Percent C.V.	1.69	9.64	3.05	7.01
Percent R.E.	11.3	14.6	18.4	14.7

TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT BILE PRECIPITATION ASSAY

Interday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/ml) 0.944	4.72	14.16	28.32
		Measured Concentrations (µg/ml)			
1	1	0.910	4.45	13.6	27.5
	2	1.01	4.46	13.9	27.1
3	1	0.870	4.32	13.1	26.9
	2	1.05	4.48	13.6	28.9
4	1	0.961	4.08	13.0	27.4
	2	0.988	4.38	13.7	28.1
Mean		0.965	4.36	13.5	27.7
S.D.		0.0661	0.15	0.354	0.737
Percent C.V.		6.85	3.44	2.63	2.67
Percent R.E.		2.21	-7.59	-4.78	-2.37

TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT BILE PRECIPITATION ASSAY

Intraday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/ml) 0.944	4.72	14.16	28.32
		Measured Concentrations (µg/ml)			
2	1	0.961	4.38	13.4	27.0
	2	0.948	4.20	13.4	28.2
	3	0.966	4.43	13.3	27.8
	4	1.03	4.49	13.6	28.8
	5	0.934	4.48	13.5	28.1
	6	1.00	4.32	14.0	27.1
Mean		0.973	4.38	13.5	27.8
S.D.		0.036	0.110	0.25	0.69
Percent C.V.		3.65	2.51	1.85	2.48
Percent R.E.		3.09	-7.13	-4.43	-1.72

TABLE 4C: PRECISION OF WR 178,460 FREE BASE RAT BILE PRECIPITATION ASSAY

Interday Precision WR 178,460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/ml) 0.860	4.30	12.84	25.68
		Measured Concentrations (µg/ml)			
1A	1	0.946	4.14	12.4	25.8
	2	1.08	4.22	12.6	25.0
3A	1	0.879	3.97	12.2	25.1
	2	0.947	4.15	12.2	26.7
4A	1	0.810	3.85	12.7	26.1
	2	0.797	3.96	13.0	26.2
Mean		0.910	4.05	12.5	25.8
S.D.		0.105	0.142	0.313	0.662
Percent C.V.		11.6	3.52	2.50	2.56
Percent R.E.		6.29	-5.41	-2.21	0.532

TABLE 4D: PRECISION OF WR 178,460 FREE BASE RAT BILE PRECIPITATION ASSAY

Intraday Precision WR 178,460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/ml) 0.856	4.28	12.84	25.68
		Measured Concentrations (µg/ml)			
2A	1	0.892	3.99	12.4	25.5
	2	0.856	3.89	12.5	26.2
	3	0.849	4.15	12.4	25.5
	4	0.900	4.09	12.4	26.5
	5	0.820	4.06	12.4	25.6
	6	0.914	3.94	12.8	24.7
Mean		0.872	4.02	12.5	25.7
S.D.		0.036	0.098	0.16	0.63
Percent C.V.		4.12	2.43	1.28	2.45
Percent R.E.		1.85	-6.07	-2.47	-0.05

TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT BILE BY PRECIPITATION

SAMPLE ID	SPIKED CONCENTRATION Range	(µg/ml)	PEAK HEIGHT RATIO REFERENCE	PEAK HEIGHT RATIO BILE	MEAN PERCENT RECOVERY
Halofantrine					
1	Extra Low	0.944	0.111	0.130	108
2			0.117	0.114	
3			0.099	0.110	
Mean (± SD)			0.109 ±0.009	0.118 ±0.011	
1	Low	4.72	0.534	0.575	105
2			0.563	0.587	
3			0.547	0.569	
Mean (± SD)			0.548 ±0.015	0.577 ±0.009	
1	Medium	14.16	1.760	1.880	103
2			1.836	1.848	
3			1.765	1.794	
Mean (± SD)			1.787 ±0.043	1.841 ±0.043	
1	High	28.32	3.537	3.867	97.8
2			3.951	3.773	
3			4.032	3.629	
Mean (± SD)			3.840 ±0.266	3.756 ±0.120	
AVERAGE MEAN RECOVERY =					104
WR 178,460					
1	Extra Low	0.856	0.126	0.126	109
2			0.141	0.133	
3			0.105	0.147	
Mean (± SD)			0.124 ±0.018	0.135 ±0.011	
1	Low	4.28	0.635	0.656	101
2			0.683	0.679	
3			0.616	0.625	
Mean (± SD)			0.645 ±0.035	0.653 ±0.027	
1	Medium	12.84	2.077	2.167	102
2			2.109	2.108	
3			1.990	2.047	
Mean (± SD)			2.059 ±0.062	2.107 ±0.060	
1	High	25.68	4.108	4.461	98.4
2			4.572	4.414	
3			4.627	4.225	
Mean (± SD)			4.436 ±0.285	4.367 ±0.125	
OVERALL AVERAGE RECOVERY =					103

LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460 (AS FREE BASES) IN RAT BILE EXTRACTION ASSAY, STUDY REPORT 17, SUPPLEMENT IV

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.

2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.

3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.

4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.

2. All chemicals are reagent grade.

3. Halofantrine hydrochloride, bottle no. BB 43807 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.

4. WR 178,460 (hydrochloride), bottle no. BK 21070, (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.

5. WR 122,455 (internal standard), bottle no. AX 26839 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.

6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).

7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).

8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).

9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

C. ASSAY CONDITIONS

1. DETECTOR

   Settings

   Wavelength: excitation - 300 nm, emission - 375 nm
   Sensitivity: high
   Range: 4
   Response: medium

   Lamp

   Ushio xenon, type UXL-155-LCA(S-LC)

2. COLUMN

   Axxiom Silica, 5 µm particle size, 4.6 x 250 mm
   (Richard Scientific, Novato, CA).

3. SOLVENT SYSTEM

   Combine and mix H_2O (800 ml) + $(NH_4)_2HPO_4$ (20 ml of 1 M $(NH_4)_2HPO_4$) + CH_3OH (3200 ml).

4. FLOW RATE

   1.0 ml/min.

5. STOCK SOLUTIONS - Solutions were stored in the -20°C freezer and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

   A. Stock Solutions

      i. HALOFANTRINE - (Halofantrine Hydrochloride).

Prep date: 3/1/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.60	0.942	50.6	methanol	104
Precision	6.24	0.942	50.0	methanol	118

*= Molecular weights of halofantrine free base/halofantrine hydrochloride

ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride).

Prep date: 3/1/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	5.60	0.924	50.6	methanol	102
Precision	6.03	0.924	52.0	methanol	107

*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

iii. WR 122,455 (Internal Standard).

Prep date: 3/4/94

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	5.20	1	25	methanol	208

B. Working Solutions

A. Halofantrine and WR 178,460 Mixed Working Solutions

1. High Concentration Working Solution: Combine 8.00 ml each of halofantrine and WR 178,460 (as free bases) stock solutions.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	8.00	100	methanol	8.32
Precision (Halofantrine)	118	8.00	100	methanol	9.44
Standard Curve (WR 178,460)	102	8.00	100	methanol	8.16
Precision (WR 178,460)	107	8.00	100	methanol	8.56

2. Low Concentration Working Solution: Combine 1.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 100 ml.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	104	2.00	100	methanol	2.08
Precision (Halofantrine)	118	2.00	100	methanol	2.36

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (WR 178,460)	102	2.00	100	methanol	2.04
Precision (WR 178,460)	107	2.00	100	methanol	2.14

B. WR 122,455 - Internal standard.

Prep date: 3/4/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	208	0.5	100	methanol	1.04

7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.

   a. Halofantrine - 6.8 min.
   b. WR 178,460 - 9.6 min.
   c. WR 122,455 (Internal Standard) - 11 min.

8. BLANK RAT BILE

   Supplied by WRAIR.

9. INJECTION VOLUME

   50 - 100 µl

10. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of $1/y_i$.

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat bile were based on the interday and intraday low point validation results (Table 3).)

    20.8 ng/ml halofantrine (free base).
    20.4 ng/ml WR 178,460 (free base).

12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

    Plasma samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. The drug is spiked with a Hamilton syringe.

13. WISP OPERATING TEMPERATURE

    Room temperature.

D. SAMPLE STORAGE

All samples are to be kept frozen at -20°C before analysis and thawed at room temperature for preparation (within 30 min.) and analysis.

E. SAMPLE PREPARATION

1. Pipet 100 µl rat bile samples into 16 X 125 silanized tubes on ice. Add 100 µl water.
2. Add 20 µl of 1.04 µg/ml WR 122,455 internal standard solution. Vortex for 30s.
3. Add 50 µl 0.1N NaOH. Vortex 30 s.
4. Add 2.0 ml methyl *t*-butyl ether. Vortex 1 min., twice. Centrifuge for 10 min. at 3000 *g*.
5. Freeze aqueous layer in dry ice/methanol bath and pour organic layer into a 13 x 100 silanized tube.
6. Repeat steps 4 and 5.
7. Evaporate to dryness. Reconstitute in 4:1 methanol/water (v/v) with a final 0.001% HCl concentration.
8. Transfer supernatant to silanized inserts and inject onto column.

F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 100 µl bile samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

Generation of Halofantrine Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (ng/ml)
00	0	0	0	0
0	0	0	0	0
2	1	2.08	2.08	20.8
3	2	2.08	4.16	41.6
4	4	2.08	8.32	83.2
5	8	2.08	16.64	166.4
6	4	8.32	33.28	333
7	8	8.32	66.56	666
8	16	8.32	133.12	1331

Generation of WR 178,460 Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (ng/ml)
00	0	0	0	0
0	0	0	0	0
2	1	2.04	2.04	20.4
3	2	2.04	4.08	40.8
4	4	2.04	8.16	81.6
5	8	2.04	16.32	163
6	4	8.16	32.64	326
7	8	8.16	65.28	653
8	16	8.16	130.56	1306

G. QUALITY CONTROL

1. Content and frequency of blanks

   No special blank was used except for the standard curve blank.

2. Pipette Calibration

   See ASOP 2C-1.1.

3. Balance Calibration

   See ASOP 2C-2.1.

H. RECOVERY

Assay recovery was assessed at four different halofantrine and WR 178,460 (as free bases) concentrations (equal to precision sample nominal concentrations) by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in bile (100 µl) samples. The bile samples were generated by spiking on ice 100 µl of blank rat

bile to which water (100 µl) has been added with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). Each bile sample was prepared as described in "Sample Preparation" above, except the internal standard was added to the transferred organic layer prior to evaporation. The reference samples (100 µl rat bile) were generated by spiking methyl *t*-butyl ether extracts of rat bile (obtained as described in "Sample Preparation" above) with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). These reference samples were then prepared by completion of the sample preparation procedure as described (addition of IS, evaporation and reconstitution).

I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 100 µl bile specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Halofantrine Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (ng/ml)
XL	2	2.36	100	47.2
Low	4	2.36	100	94.4
Med.	4	9.44	100	378
Hi	10	9.44	100	944

Generation of WR 178,460 Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (ng/ml)
XL	2	2.14	100	42.8
Low	4	2.14	100	85.6
Med.	4	8.56	100	342
Hi	10	8.56	100	856

J. RESULTS

1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

2. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

   Results for this evaluation appear in Table 2.

3. LOW POINT VALIDATION

   Results for this evaluation appear in Table 3.

3. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 4A-D.

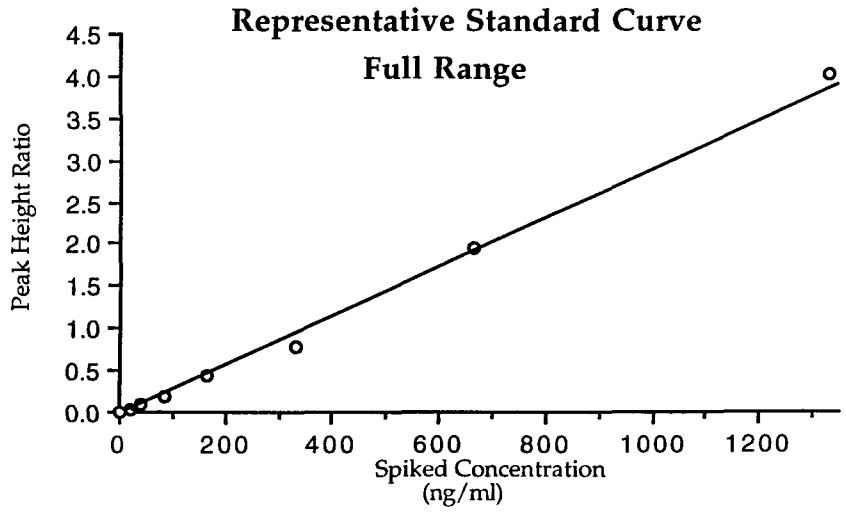
4. RECOVERY

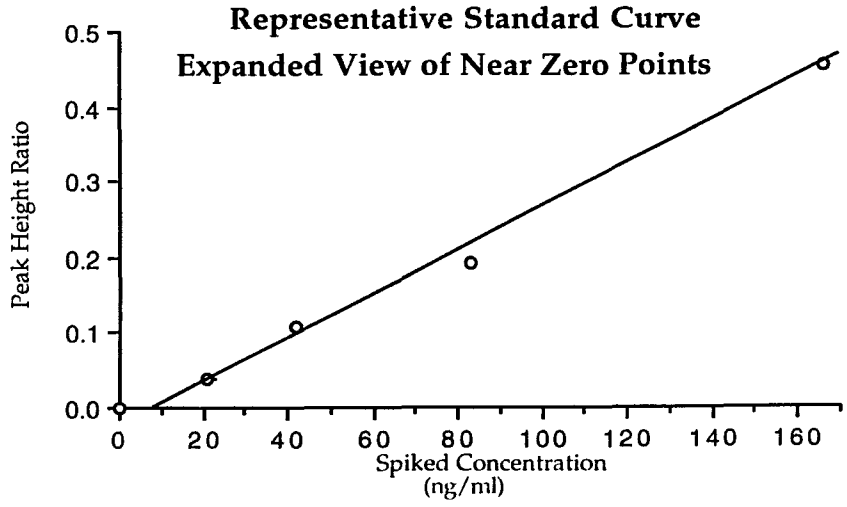
   Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR HALOFANTRINE (FREE BASE) RAT BILE EXTRACTION ASSAY, STUDY REPORT 17, SUPPLEMENT NO. 4

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
2.08	20.8	0.040	22.7
4.16	41.6	0.110	46.8
8.32	83.2	0.193	75.3
16.64	166	0.453	165
33.28	333	0.774	275
66.56	666	1.957	682
133.12	1331	4.025	1390

Regression equation:***
$y = 0.00291x - 0.00261$, $r^2 = 0.9920$



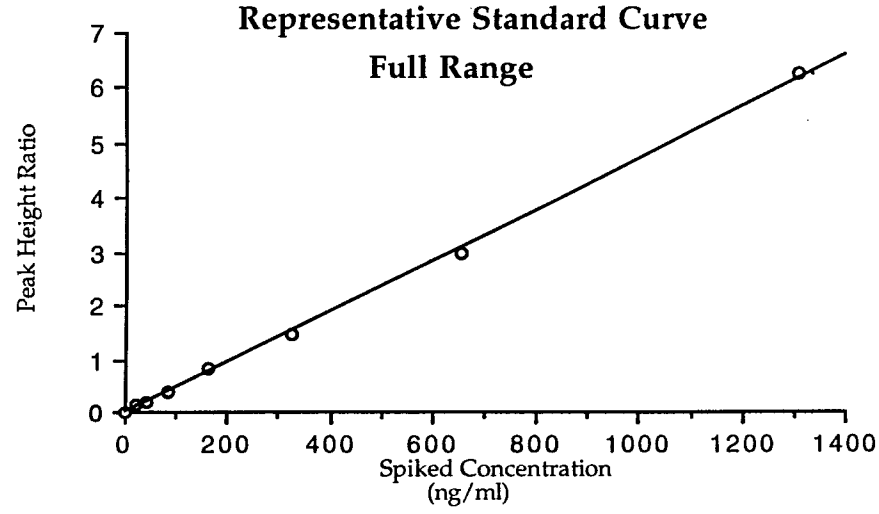


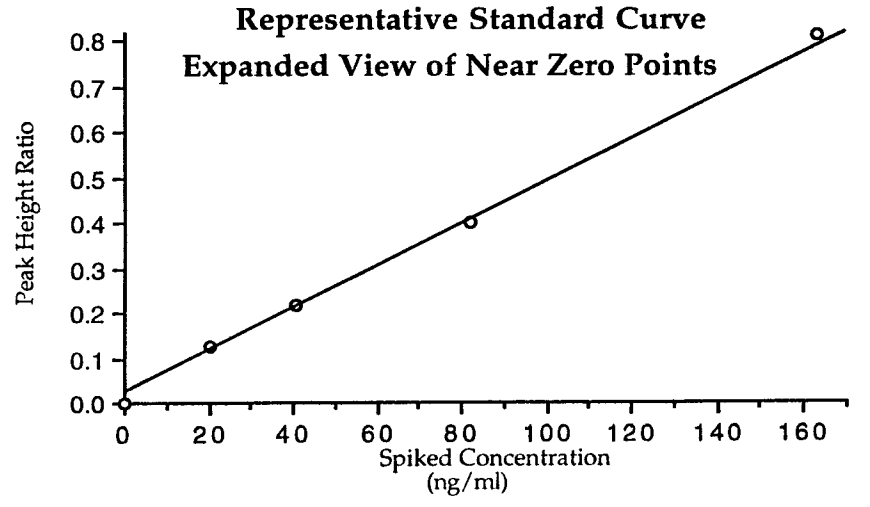
* Into 100 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR WR 178,460 (FREE BASE) RAT BILE EXTRACTION ASSAY, STUDY REPORT 17, SUPPLEMENT NO. 4

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
2.04	20.4	0.124	21.3
4.08	40.8	0.216	40.9
8.16	81.6	0.400	80.2
16.32	163	0.813	169
32.64	326	1.481	311
65.28	653	3.004	637
130.56	1306	6.264	1330

Regression equation:*** $y = 0.00468x + 0.0245$, $r^2 = 0.9991$





* Into 100 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 2: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE AND WR 178,460 AS FREE BASES RAT BILE EXTRACTION ASSAY, STUDY REPORT 17B, SUPPLEMENT IV

Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
Halofantrine				
7/15/94	1(Intraday)	0.0038786	-0.0225222	0.9990543
7/19/94	2(Interday 1)	0.00334147	-0.0065534	0.99197203
7/21/94	3(Interday 2)	0.00389226	-0.0694891	0.99052267
8/5/94	4(Interday 3)	0.00290851	-0.0260724	0.992005
WR 178460				
7/15/94	1A(Intraday)	0.00487798	-0.0318817	0.99871791
7/19/94	2A(Interday 1)	0.00490082	-0.0106928	0.99079816
7/21/94	3A(Interday 2)	0.00500511	-0.0424384	0.99768128
8/5/94	4A(Interday 3)	0.00467863	0.02454114	0.99907353

Halofantrine Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (ng/ml) 20.8	41.6	83.2	166	333	666	1331
	Back Calculated Concentration (ng/ml)						
1	22.9	43.6	80.4	139	358	674	BC
2	BC	51.3	81.6	139	324	622	1420
3	22.7	46.8	75.3	165	275	682	1390
4	22.8	40.9	77.7	169	320	655	1360
Mean	22.8	45.7	78.8	153	319	658	1390
S.D.	0.100	4.47	2.82	16.2	34.1	26.7	30.0
Percent C.V.	0.439	9.80	3.58	10.6	10.7	4.05	2.16
Percent R.E.	9.62	9.74	-5.35	-7.83	-4.13	-1.16	4.43

WR 178460 Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (ng/ml) 20.4	40.8	81.6	163	326	653	1306
	Back Calculated Concentration (ng/ml)						
1A	23.8	47.1	71.8	152	308	592	1410
2A	bc	45.2	79.6	157	311	627	1350
3A	21.3	40.9	80.2	169	311	637	1330
4A	24	35.9	80.7	165	310	651	1330
Mean	23.0	42.3	78.1	161	310	627	1355
S.D.	1.50	4.98	4.21	7.68	1.41	25.2	37.9
Percent C.V.	6.53	11.8	5.39	4.77	0.46	4.02	2.79
Percent R.E.	12.9	3.62	-4.32	-1.38	-4.91	-4.02	3.75

TABLE 3: LOW POINT VALIDATION OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) RAT BILE EXTRACTION ASSAY

Spiked Concentration	HALOFANTRINE (free base) (20.8 ng/ml)		WR 178,460 (free base) (20.4 ng/ml)	
	Measured Concentrations (ng/ml)			
	Interday	Intraday	Interday	Intraday
	22.9	bc	23.8	bc
	bc	23.1	bc	20.4
	22.7	22.0	21.3	21.5
		24.1		19.1
		22.0		21.3
		23.7		20.4
Mean	22.8	23.0	22.6	20.5
Standard Deviation	0.141	0.963	1.77	0.950
Percent CV	0.62	4.19	7.84	4.63
Percent Error	9.62	10.5	10.5	0.69

TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT BILE EXTRACTION ASSAY

Interday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (ng/ml) 47.2	94.4	378.0	944
		Measured Concentrations (ng/ml)			
2	1	59.7	86.4	382	bc
	2	48.0	83.4	380	bc
3	1	49.2	90	344	833
	2	50.7	88.5	334	850
4	1	40.3	98.7	349	834
	2	39.6	98	336	898
Mean		47.9	90.8	354	854
S.D.		7.42	6.23	21.5	30.5
Percent C.V.		15.5	6.86	6.07	3.57
Percent R.E.		1.52	-3.78	-6.31	-9.56

TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT BILE EXTRACTION ASSAY

Intraday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (ng/ml) 47.2	94.4	378	944
		Measured Concentrations (ng/ml)			
1	1	53.0	79.8	355	1000
	2	36.2	77.7	361	925
	3	43.2	88.8	363	917
	4	48.6	87.5	337	911
	5	46.3	84.4	371	928
	6	40.6	89.6	382	901
Mean		44.7	84.6	362	930
S.D.		5.97	4.93	15.2	35.5
Percent C.V.		13.4	5.83	4.21	3.82
Percent R.E.		-5.40	-10.3	-4.37	-1.45

TABLE 4C: INTERDAY PRECISION OF WR 178,460 FREE BASE RAT BILE EXTRACTION ASSAY

Interday Precision WR 178,460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (ng/ml) 42.8	85.6	342	856
		Measured Concentrations (ng/ml)			
2A	1	44.4	79.9	317	806
	2	40.3	81.1	336	799
3A	1	51.0	93.0	332	795
	2		89.8	351	825
4A	1	33.0	83.9	320	796
	2	36.6	80.7	314	824
Mean		41.1	84.7	328	808
S.D.		6.99	5.43	14.1	13.7
Percent C.V.		17.0	6.41	4.28	1.70
Percent R.E.		-4.07	-1.01	-4.00	-5.67

TABLE 4D: INTRADAY PRECISION OF WR 178,460 FREE BASE RAT BILE EXTRACTION ASSAY

Intraday Precision WR 178,460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (ng/ml) 42.8	85.6	342	856
		Measured Concentrations (ng/ml)			
1A	1	47.1	63.7	333	889
	2	37.5	88.3	344	851
	3	46.3	86.1	326	836
	4	43.6	88.5	334	828
	5	49.0	83.6	341	846
	6	45.5	86.1	360	832
Mean		44.8	82.7	340	847
S.D.		4.01	9.49	11.8	22.3
Percent C.V.		8.94	11.5	3.48	2.63
Percent R.E.		4.75	-3.37	-0.68	-1.05

TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT BILE BY EXTRACTION

SAMPLE ID	SPIKED CONCENTRATION Range	(ng/ml)	PEAK HEIGHT RATIO REFERENCE	PEAK HEIGHT RATIO BILE	MEAN PERCENT RECOVERY
Halofantrine					
1	Extra Low	47.2	0.162	0.100	81.5
2			0.159	0.144	
3			0.161	0.149	
Mean (± SD)			0.161 ±0.002	0.131 ±0.027	
1	Low	94.4	0.309	0.209	68.0
2			0.302	0.215	
3			0.329	0.215	
Mean (± SD)			0.313 ±0.014	0.213 ±0.003	
1	Medium	378	1.389	0.975	64.6
2			1.429	0.89	
3			1.474	0.908	
Mean (± SD)			1.431 ±0.043	0.924 ±0.045	
1	High	944	3.357	2.274	67.8
2			3.676	2.455	
3			3.58	2.463	
Mean (± SD)			3.538 ±0.164	2.397 ±0.107	
OVERALL AVERAGE RECOVERY =					70.5
WR 178,460					
1	Extra Low	42.8	0.221	0.224	90.1
2			0.214	0.191	
3			0.241	0.194	
Mean (± SD)			0.225 ±0.014	0.203 ±0.018	
1	Low	85.6	0.391	0.381	90.0
2			0.356	0.33	
3			0.412	0.332	
Mean (± SD)			0.386 ±0.028	0.348 ±0.029	
1	Medium	342	1.682	1.577	90.6
2			1.718	1.558	
3			1.734	1.515	
Mean (± SD)			1.711 ±0.027	1.550 ±0.032	
1	High	856	4.051	3.771	89.7
2			4.382	3.89	
3			4.298	3.757	
Mean (± SD)			4.244 ±0.172	3.806 ±0.073	
OVERALL AVERAGE RECOVERY =					90.1

LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460 AS FREE BASES IN RAT LIVER HOMOGENATE ASSAY, STUDY REPORT 17B, SUPPLEMENT V

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Halofantrine hydrochloride, bottle no. BB 43807 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
4. WR 178,460 (hydrochloride), bottle no. BK 21070, (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
5. WR 122,455 (internal standard), bottle no. AX 26839 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

C. ASSAY CONDITIONS

1. DETECTOR

Settings
Wavelength: excitation - 300 nm, emission - 375 nm
Sensitivity: high
Range: 4
Response: medium
Lamp: Ushio xenon, type UXL-155-LCA(S-LC)

2. COLUMN

Axxiom Silica, 5 µm, 4.6 x 250 mm (Richard Scientific, Novato, CA).

3. SOLVENT SYSTEM

Combine and mix H_2O (800 ml) + $(NH_4)_2HPO_4$ (20 ml of 1 M $(NH_4)_2HPO_4$) + CH_3OH (3200 ml).

4. FLOW RATE

1.0 ml/min.

5. SOLUTIONS - Solutions were stored in the -20°C freezer and checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Storage bottles were amber or covered with aluminum foil.

A. Stock Solutions

i. HALOFANTRINE - (Halofantrine Hydrochloride).

Prep date: 5/9/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	14.3	0.942	25.0	methanol	539
Precision	14.2	0.942	25.0	methanol	535

*= Molecular weights of halofantrine free base/halofantrine hydrochloride

ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride).

Prep date: 5/9/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	14.6	0.924	25.0	methanol	540
Precision	14.5	0.924	25.0	methanol	536

*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

iii. WR 122,455 (Internal Standard).

Prep date: 3/4/94

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	5.20	1	25	methanol	208

B. Working Solutions

i. Halofantrine and WR 178,460 Mixed Working Solutions

a. LOW CONCENTRATION WORKING SOLUTION - Combine halofantrine and WR 178,460 (as free bases) stock standard curve solutions.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve (Halofantrine)	540	5.00	25.0	methanol	108
Standard Curve (WR 178,460)	539	5.00	25.0	methanol	108
Precision (Halofantrine)	535	5.00	25.0	methanol	107
Precision (WR 178,460)	536	5.00	25.0	methanol	107

b. HIGH CONCENTRATION WORKING SOLUTION - Combine halofantrine and WR 178,460 (as free bases) stock solutions.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (ng/ml)
Standard Curve (Halofantrine)	108	8.00	20.0	methanol	216
Standard Curve (WR 178,460)	108	8.00	20.0	methanol	216
Precision (Halofantrine)	107	8.00	20.0	methanol	214
Precision (WR 178,460)	107	8.00	20.0	methanol	214

ii. WR 122,455 - Internal standard.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (part)	QS Volume (part)	Solvent	Conc. (µg/ml)
Internal std.	208	0.5	10.5	methanol	9.90

6. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.
   a. Halofantrine - 7.1 min.
   b. WR 178,460 - 10.8 min.
   c. WR 122,455 (Internal Standard) - 12.8 min.

7. BLANK RAT LIVER HOMOGENATE
   Supplied by WRAIR.

8. INJECTION VOLUME
   5 µl

9. QUANTITATION
   By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of $1/y_i$.

10. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat liver homogenate were based on the interday and intraday low point validation results (Table 3).)
    0.540 µg/ml halofantrine (free base).
    0.540 µg/ml WR 178,460 (free base).

11. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT
    Liver homogenate samples and internal standard spiking volumes were measured with a calibrated (SOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. Drugs are spiked with Hamilton syringes.

12. WISP OPERATING TEMPERATURE
    Room temperature.

13. LIVER HOMOGENIZATION
    Rat liver (1 g) is combined with 5 ml of buffer (combine 245 ml H_2O, 5 ml HCl and 250 ml methanol). Samples are homogenized in a Waring Commercial (Waring Products, New Hartford, CN) blender in a Mini Container.

D. SAMPLE STORAGE

All samples are to be kept frozen at -70°C before analysis and thawed at room temperature for preparation (within 30 min.) and analysis.

E. SAMPLE PREPARATION

1. Pipet 0.200 ml rat liver homogenate samples into 13 X 100 silanized tubes.

2. Add 0.6 ml CH_3CN. Vortex 1 min.

3. Add 40 µl of 10.0 µg/ml WR 122,455 internal standard solution. Vortex for 1 min.

4. Centrifuge at 3000 *g* for 10 min.

5. Transfer supernatant to silanized inserts and inject 5 µl onto column.

F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike 0.2 ml rat liver homogenate standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 0.200 ml liver homogenate samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

Generation of Halofantrine and WR 178,460 Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (µg/ml)
00	0		0	0
0	0		0	0
1	1.00	108	108	0.540
2	2.00	108	216	1.08
3	4.00	108	432	2.16
4	8.00	108	864	4.32
5	16.0	108	1728	8.64
6	16.0	216	3456	17.3
7	32.0	216	6912	34.6
8	64.0	216	13824	69.1

G. QUALITY CONTROL

1. Content and frequency of blanks

   No special blank was used except for the standard curve blank.

2. Pipette Calibration

   See SOP 2C-1.1.

3. Balance Calibration

   See SOP 2C-2.1.

H. RECOVERY

Assay recovery was assessed at four different concentrations by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in liver homogenate samples. Liver homogenate (200 µl) and reference (200 µl water) samples were spiked with corresponding amounts of halofantrine and WR 178,460 (as free bases) prior to addition of acetonitrile precipitant. Each liver homogenate and reference sample was prepared as described in "Sample Preparation" (Section E), except the internal standard was added to 500 µl of the transferred supernatant (after step 5, not in step 3).

I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 200 µl liver homogenate specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Halofantrine and WR 178,460 Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (µl)	Precision Sample Nominal Concentration (µg/ml)
Hi	12	214	200	25.0
Med.	6	107	200	5.35
Low	10	107	200	1.07

J. RESULTS

1. STANDARD CURVE

   Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

2. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

   Results for this evaluation appears in Table 2.

3. LOW POINT VALIDATION

   Results for this evaluation appears in Table 3.

4. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 4A-D.

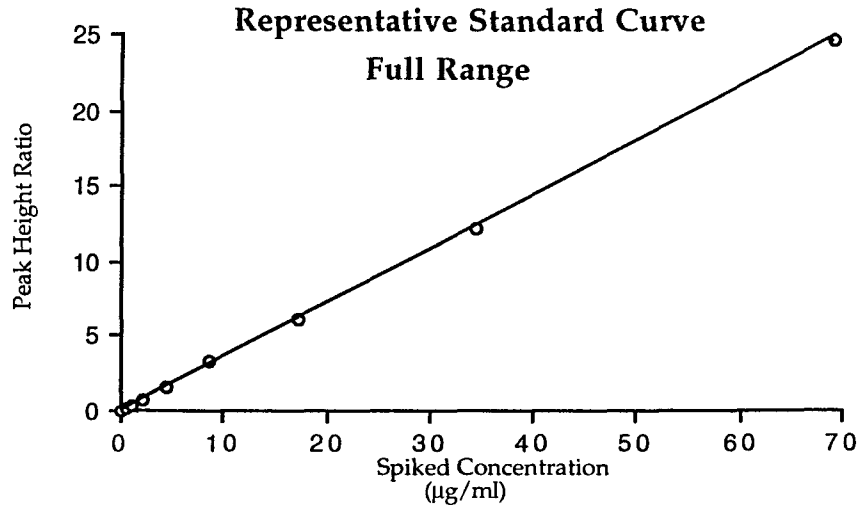
5. RECOVERY

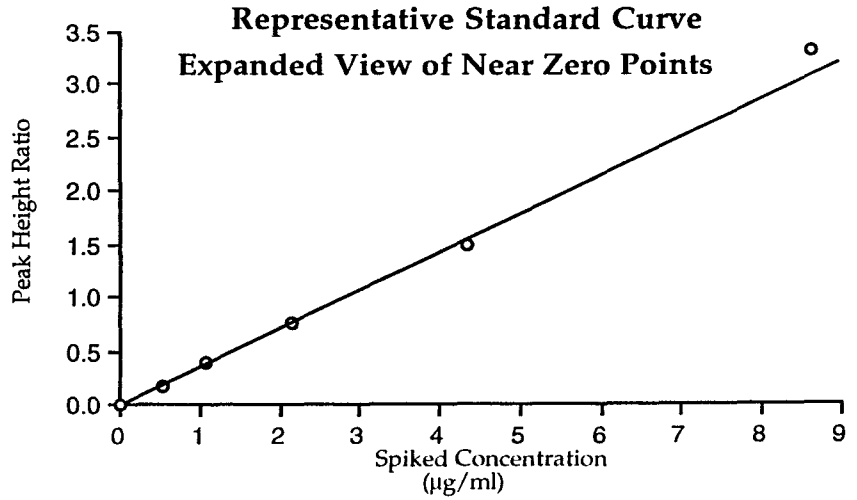
   Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR HALOFANTRINE (FREE BASE) RAT LIVER HOMOGENATE ASSAY, STUDY REPORT 17, SUPPLEMENT NO. V

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (µg/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (µg/ml)
0	0	-	-
108	0.54	0.169	0.506
216	1.08	0.400	1.15
432	2.16	0.748	2.12
864	4.32	1.494	4.20
1728	8.64	3.326	9.31
3456	17.3	6.121	17.1
6912	34.6	12.324	34.4
13824	69.1	24.708	69.0

Regression equation:***
$y = 0.358x - 0.0124$, $r^2 = 0.9995$



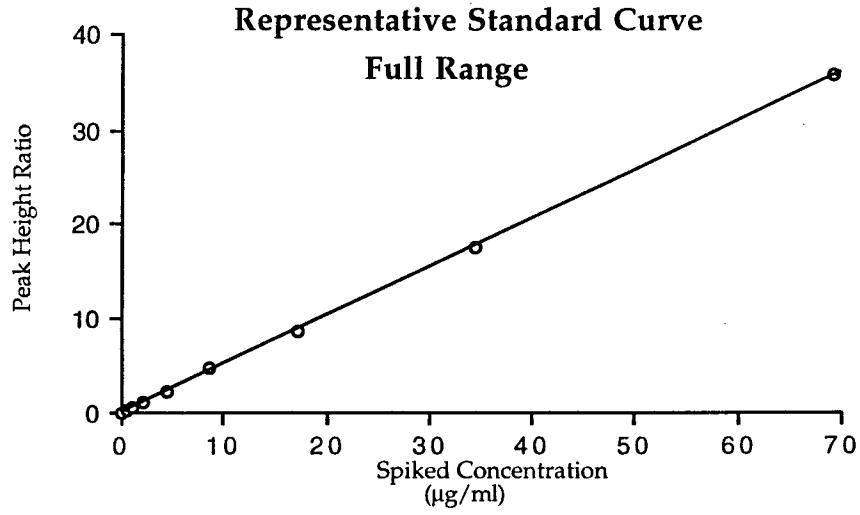


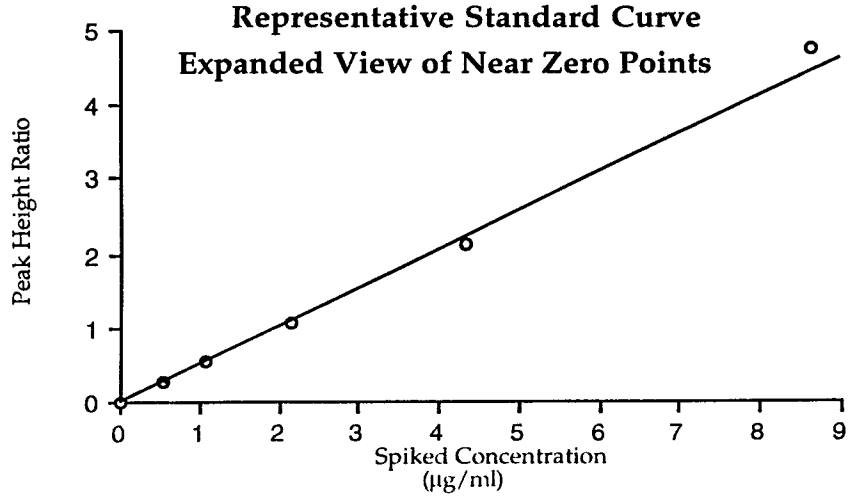
* Into 200 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR WR 178,460 (FREE BASE) RAT LIVER HOMOGENATE ASSAY, STUDY REPORT 17, SUPPLEMENT NO. V

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (µg/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (µg/ml)
0	0	-	-
108	0.540	0.297	0.561
216	1.08	0.562	1.08
432	2.16	1.093	2.11
864	4.32	2.135	4.14
1728	8.64	4.766	9.26
3456	17.3	8.629	16.8
6912	34.6	17.635	34.3
13824	69.1	35.770	69.6

Regression equation:***
$y = 0.514x - 0.0087$, $r^2 = 0.9994$





* Into 200 µl of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 2: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE AND WR 178,460 AS FREE BASES RAT LIVER ASSAY, STUDY REPORT 17B, SUPPLEMENT V

Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
Halofantrine				
5/12/94	1(Intraday)	0.358405524	-0.01242626	0.999479418
5/19/94	2(Interday 1)	0.355267367	0.012544907	0.999903401
5/20/96	3(Interday 2)	0.364534322	0.014512231	0.99832086
5/22/96	4(Interday 3)	0.353792812	0.011080767	0.999082954
WR 178460				
5/12/94	1A(Intraday)	0.513864726	0.00868	0.999386942
5/19/94	2A(Interday 1)	0.508007617	0.015582886	0.999764575
5/20/96	3A(Interday 2)	0.521074272	0.025879712	0.998951121
5/22/96	4A(Interday 3)	0.507207281	0.000278691	0.998773148

Halofantrine Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml)							
	0.540	1.08	2.16	4.32	8.64	17.3	34.6	69.1
	Back Calculated Concentration (µg/ml)							
1	0.506	1.15	2.12	4.2	9.31	17.1	34.4	69.0
2	0.550	1.07	2.12	4.26	8.92	17.2	34.5	69.1
3	0.649	1.01	2.02	4.09	8.48	17.9	36.4	67.4
4	0.554	1.04	2.22	4.17	9.06	17.3	33.2	70.3
Mean	0.565	1.07	2.12	4.18	8.94	17.4	34.6	69.0
S.D.	0.0602	0.060	0.0816	0.071	0.348	0.359	1.32	1.19
Percent C.V.	10.7	5.64	3.85	1.69	3.89	2.07	3.82	1.73
Percent R.E.	4.58	-1.16	-1.852	-3.24	3.50	0.434	0.072	-0.217

WR 178460 Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml)							
	0.540	1.08	2.16	4.32	8.64	17.3	34.6	69.1
	Back Calculated Concentration (µg/ml)							
1A	0.561	1.08	2.11	4.14	9.26	16.8	34.3	69.6
2A	0.526	1.11	2.13	4.22	9.07	17.2	34.3	69.3
3A	0.618	0.994	2.11	4.14	8.52	17.7	36.1	67.7
4A	0.534	1.09	2.17	4.23	9.13	17.2	32.9	70.6
Mean	0.560	1.07	2.13	4.18	9.00	17.2	34.4	69.3
S.D.	0.0416	0.0512	0.0283	0.0492	0.326	0.369	1.31	1.20
Percent C.V.	7.44	4.79	1.33	1.18	3.63	2.14	3.81	1.74
Percent R.E.	3.66	-1.06	-1.39	-3.18	4.11	-0.434	-0.578	0.289

TABLE 3: LOW POINT VALIDATION OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) RAT LIVER ASSAY

Spiked Concentration	HALOFANTRINE (free base) (0.540 µg/ml)		WR 178,460 (free base) (0.540 µg/ml)	
	Measured Concentrations (µg/ml)			
	Interday	Intraday	Interday	Intraday
	0.550	0.477	0.526	0.530
	0.649	0.559	0.618	0.570
	0.554	0.590	0.534	0.639
		0.514		0.509
		0.618		0.564
		0.539		0.568
Mean	0.584	0.550	0.559	0.563
Standard Deviation	0.056	0.051	0.051	0.044
Percent C.V.	9.59	9.30	9.11	7.88
Percent R.E.	8.21	1.76	3.58	4.32

TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT LIVER ASSAY

Interday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/ml) 1.07	5.35	24.6
		Measured Concentrations (µg/ml)		
2	1	1.13	5.36	26.0
	2	1.13	5.30	26.5
3	1	1.06	5.17	25.3
	2	0.983	5.28	25.6
4	1	1.04	5.71	25.5
	2	1.11	5.34	27.0
Mean		1.08	5.36	26.0
S.D.		0.0586	0.184	0.655
Percent C.V.		5.45	3.43	2.52
Percent R.E.		0.514	0.187	5.62

TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT LIVER ASSAY

Intraday Precision Halofantrine (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/ml) 1.07	5.35	24.6
		Measured Concentrations (µg/ml)		
1	1	1.21	5.58	26.4
	2	1.22	5.57	27.1
	3	1.2	5.27	26.2
	4	1.23	5.43	27.3
	5	1.12	4.98	26.5
	6	1.11	5.05	25.6
Mean		1.18	5.31	26.5
S.D.		0.053	0.258	0.62
Percent C.V.		4.46	4.86	2.33
Percent R.E.		10.4	-0.685	7.79

TABLE 4C: PRECISION OF WR 178,460 FREE BASE RAT LIVER ASSAY

Interday Precision WR 178,460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/ml) 1.07	5.35	24.6
		Measured Concentrations (µg/ml)		
2A	1	1.20	5.43	23.7
	2	1.21	5.38	24.0
3A	1	1.01	5.20	23.1
	2	0.992	5.24	23.3
4A	1	1.14	5.75	23.2
	2	1.17	5.42	24.5
Mean		1.12	5.40	23.6
S.D.		0.0958	0.195	0.543
Percent C.V.		8.55	3.61	2.30
Percent R.E.		4.70	0.997	-3.93

TABLE 4D: PRECISION OF WR 178,460 FREE BASE RAT LIVER ASSAY

Intraday Precision WR 178,460 (Free Base)

Validation Run	QC Sample No.	Spiked Concentrations (µg/ml) 1.07	5.35	24.6
		Measured Concentrations (µg/ml)		
1A	1	1.12	5.62	23.8
	2	1.15	5.56	24.6
	3	1.20	5.15	23.5
	4	1.16	5.37	24.7
	5	1.09	4.96	23.7
	6	1.07	5.03	23.2
Mean		1.13	5.28	23.9
S.D.		0.048	0.277	0.60
Percent C.V.		4.23	5.25	2.53
Percent R.E.		5.76	-1.28	-2.78

TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT LIVER HOMOGENATE BY PRECIPITATION

SAMPLE ID	SPIKED CONCENTRATION Range	(µg/ml)	PEAK HEIGHT RATIO REFERENCE	LIVER HOMOGENATE	MEAN PERCENT RECOVERY
Halofantrine					
1	High	24.6	6.698	7.384	100
2			7.189	6.939	
3			6.939	6.591	
Mean (± SD)			6.942 ±0.246	6.971 ±0.397	
1	Medium	5.35	1.487	1.414	93.3
2			1.669	1.486	
3			1.515	1.456	
Mean (± SD)			1.557 ±0.098	1.452 ±0.036	
1	Low	1.07	0.270	0.301	103.6
2			0.308	0.301	
3			0.295	0.302	
Mean (± SD)			0.291 ±0.019	0.301 ±0.001	
OVERALL AVERAGE RECOVERY =					99.1
WR 178,460					
1	High	24.6	8.725	9.330	97.8
2			9.360	8.790	
3			8.993	8.370	
Mean (± SD)			9.026 ±0.319	8.830 ±0.481	
1	Medium	5.35	1.944	1.885	95.0
2			(2.721)bc	1.932	
3			2.035	1.855	
Mean (± SD)			1.990 ±0.064	1.891 ±0.039	
1	Low	1.07	0.403	0.362	90.9
2			0.407	0.380	
3			0.442	0.396	
Mean (± SD)			0.417 ±0.021	0.379 ±0.017	
OVERALL AVERAGE RECOVERY =					94.6

bc = unacceptable chromatogram, data not used in calculations.

LABORATORY METHODOLOGY FOR WR 6026 AND WR 211,789 (AS FREE BASES) PLASMA ASSAY,* STUDY REPORT 18

presented in mid term report

* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

LABORATORY METHODOLOGY FOR STUDY REPORT 19: MEFLOQUINE (FREE BASE) HUMAN BLOOD ASSAY

presented in mid term report

LABORATORY METHODOLOGY FOR STUDY REPORT 20: ARTELINIC ACID HUMAN PLASMA ASSAY

A. INSTRUMENTS

1. Waters Associates WISP 710B (Waters Associates, Milford, MA), or equivalent.
2. Shimadzu LC 6A (Shimadzu Scientific Instruments, Inc., Columbia, MD), or equivalent.
3. Kratos Spectroflow 783 UV Absorbance Detector (Kratos Analytical Instruments, Ramsey, NJ), or equivalent.
4. Hewlett-Packard Integrator 3390A (Hewlett-Packard Co., Santa Clara, CA), or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Artelinic acid, WR 255663AK (Walter Reed Army Institute of Research, Washington D.C.), bottle number BM04131, expiration date not available.
4. Meclofenamic acid (Internal Standard), (Mylan Pharmaceuticals, and WV), purified by ET Lin.
5. Methanol (Fisher Scientific, Fair Lawn, NJ).
6. Acetonitrile (Fisher Scientific, Fair Lawn, NJ).
7. 85% Phosphoric acid (Fisher Scientific, Fair Lawn, NJ).
8. Type 1 reagent grade water: prepared with a Nanopure II system, Barnstead Co., Boston, MA).

*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

C. ASSAY CONDITIONS

1. DETECTOR

   Settings
   Wavelength: 236 nm
   Sensitivity: 0.008 aufs

   Lamp: Applied Biosystems deuterium, model 120-LC

2. COLUMN

   Axxiom ODS, 5 µm particle size, 4.6 x 250 mm (Richard Scientific, Novato, CA).

3. SOLVENT SYSTEM

   CH_3CN/50 mM $NH_4H_2PO_4$ (1:1, v/v), pH adjusted to 5.00 with H_3PO_4

4. FLOW RATE

   1.0 ml/min

5. STOCK SOLUTIONS - Solutions were stored in a -20°C freezer and checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds and internal standard (solutions are discarded when a more than 10% change in the ratio is observed or by 6 months after the preparation date).

   a. ARTELINIC ACID (Standard Curve Stock Solution)

      10.035 mg of artelinic acid dissolved in and q.s. to 50.0 ml with methanol.

      Purity factor = 0.9487

      Conc. of artelinic acid stock = 0.190 mg/ml

   b. ARTELINIC ACID (Control Stock Solution)

      12.594 mg of artelinic acid dissolved in and q.s. to 50.00 ml with methanol.

      Purity factor = 0.9487

      Conc. of artelinic acid stock = 0.239 mg/ml

*Report No. 703 titled "Assay of 10-O-(4'-Carboxybenzyl)dihydroartemisinin Hemihydrate, 4-(10'-Dihydro-artemisininoxymethyl)benzoic Acid Hemihydrate (Artelinic Acid), WR-255663AK, BM04131," p. 9.

c. INTERNAL STANDARD: Meclofenamic acid

2.945 mg of meclofenamic acid dissolved in 50.0 ml of methanol.

Conc. of meclofenamic acid stock = 58.9 µg/ml

6. WORKING SOLUTIONS - Solutions were stored in a 4°C refrigerator and discarded when stock solutions were discarded or by 6 months after the preparation date.

a. ARTELINIC ACID (Standard Curve Working Solution)

Dilute 10.0 ml of 0.190 mg/ml artelinic acid stock solution to 24.0 ml with methanol.

Conc. of artelinic acid working solution = 79.3 µg/ml

Dilute 1.0 ml of 79.3 µg/ml artelinic acid working solution to 16 ml with methanol.

Conc. of artelinic acid working solution = 4.96 µg/ml

b. ARTELINIC ACID (Control Working Solution)

Dilute 5.00 ml of 0.239 mg/ml artelinic acid stock solution to 15.0 ml with methanol.

Conc. of artelinic acid working solution = 79.7 µg/ml

Dilute 1.0 ml of 79.7 µg/ml artelinic acid working solution to 16 ml with methanol.

Conc. of artelinic acid working solution = 4.98 µg/ml

c. MECLOFENAMIC ACID (Internal standard).

Dilute 1.0 ml of 58.9 µg/ml meclofenamic acid stock solution to 18 ml with methanol.

Conc. of meclofenamic acid working solution = 3.27 µg/ml

7. RETENTION TIMES (subject to change depending on temperature and column performance).

a. Artelinic acid - 20 min

b. Meclofenamic acid (Internal Standard) - 17 min

8. BLANK PLASMA

Human plasma (CPD or CPDA-1 as anticoagulant) was obtained from San Francisco Irwin Memorial Blood Bank.

9. INJECTION VOLUME

   60 µl

10. QUANTITATION

    By peak height ratio of drug relative to internal standard peak.

11. MINIMUM QUANTITATION LIMIT OF METHOD

    The minimum quantitation limit, 5.23 ng/ml for artelinic acid, was determined as the artelinic acid concentration at which the signal to noise ratio was at least 3 to 1.

12. SAMPLE VOLUME MEASUREMENT

    Plasma sample volumes were measured with a 1000 µl Gilson Pipetman. See SOP 3-4 for calibration procedure.

13. WISP OPERATING TEMPERATURE

    Room temperature.

14. CARTRIDGE CONDITIONING

    QUATERNARY AMINOPROPYL (SAX) ION EXCHANGE CARTRIDGE (500 mg)

    a. Wash cartridge with 3 ml methanol.
    b. Wash with 2 ml 0.1 M HCl.
    c. Wash with 3 ml H_2O.
    d. Wash with 6 ml 0.1 M NaH_2PO_4, pH 7.0.

    NH_2 ION EXCHANGE CARTRIDGE (500 mg)

    Same as SAX cartridge conditioning, except:

    b. Wash with 6 ml 0.1 M HCl.
    d. Wash with 6 ml 0.1 M NaH_2PO_4, pH 7.01.

D. SAMPLE STORAGE

All samples must be kept frozen at -80°C before analysis and thawed at room temperature for preparation and analysis.

E. SAMPLE PREPARATION

SAX CARTRIDGE ELUTION

1. Pipette 1 ml plasma into culture tube.

2. Spike standard curve samples with 00, 0, 1, 2, or 8 µl of 4.96 µg/ml artelinic acid working solution or 1, 2, 4, 8, or 16 µl of 79.3 µg/ml artelinic acid working solution to make a standard curve. This procedure is equivalent to addition of 4.96, 9.92, 19.8, 39.7, 79.3, 159, 317, 635, or 1270 ng of artelinic acid to each tube. Since 1 ml of biological sample is assayed, these amounts correspond to artelinic acid concentrations of 00, 0, 4.96, 9.92, 19.8, 39.7, 79.3, 159, 317, 635, or 1270 ng/ml.

3. Add 50 µl of internal standard solution (3.27 µg/ml meclofenamic acid). Vortex 10 s.

4. Add 2 ml acetonitrile. Vortex for 1 min. Centrifuge for 10 min at 3000*g*.

5. Transfer supernatant into clean 12x75 mm tubes.

6. Evaproate to 200 µl under nitrogen.

7. Add 1 ml water. Vortex for 1 min.

8. Pour sample onto pre-conditioned 500 mg SAX cation-exchange cartridge.

9. Wash cartridge with 3 ml of water followed by 3 ml of acetonitrile by gravity elution.

10. Wash cartridge with 0.5 ml of 0.5 M formic acid in acetonitrile by gravity elution.

11. Elute sample with 2 ml of 0.5 M formic acid in acetonitrile by gravity elution.

12. Evaporate elutent to dryness under nitrogen.

13. Reconstitute sample in 200 µl of 50% acetonitrile, transfer to WISP vial and inject 60 µl onto the HPLC column.

NH_2 CARTRIDGE ELUTION: SAME AS ABOVE EXCEPT:

8. Pour sample onto pre-conditioned 500 mg $\underline{NH_2}$ cation-exchange cartridge.

9. Wash cartridge with 3 ml of water followed by 3 ml of acetonitrile by gravity elution. Completely dry cartridge on Vacelut.

*00 = Sample with no drug and no internal standard.
**0 = Sample with no drug but with internal standard.

10. Wash cartridge twice with 0.5 ml of 0.5 M formic acid in acetonitrile by gravity elution. Completely dry cartridge on Vacelut.

11. Elute sample with three 0.5 ml aliquots of 0.5 M formic acid in acetonitrile by gravity elution.

13. Reconstitute sample in 200 µl of 50% acetonitrile, transfer to WISP vial and inject 30 µl onto the HPLC column.

F. QUALITY CONTROL

1. CONTENT AND FREQUENCY OF BLANKS

   No special blank sample was assayed, except for the standard curve and blanks.

2. PIPETTE CALIBRATION

   See SOP 3-4.1.

3. BALANCE CALIBRATION

   See SOP 3-19.1

G. GENERATION OF PRECISION SAMPLES

Precision samples were made and assayed with calibration standards. Samples for precision analysis were prepared by spiking blank 1 ml plasma specimens with artelinic acid control working solution to make final artelinic acid concentrations corresponding to 9.96, 39.8, 398, or 797 ng/ml. See table.

Generation of Precision Samples (NH_2)

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	1	9.50	1	9.50
Low	4	9.50	1	38.0
Med.	4	95.0	1	380
Hi	8	95.0	1	760

Generation of Precision Samples (SAX)

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	4.98	1	9.96
Low	8	4.98	1	39.8
Med.	5	79.7	1	398
Hi	10	79.7	1	797

H. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at three different concentrations by comparing the artelinic acid to internal standard peak height ratios in solvent to the peak height ratios in plasma. Plasma (1 ml) and solvent (methanol) samples were spiked with corresponding amounts of artelinic acid. Each plasma sample was prepared as described in "Sample Preparation" (Section E), except the internal standard was added after the eluents were collected (step 11) and the solvent samples were not extracted.

I. GENERATION OF FREEZE THAW SAMPLES

The effect of repeated freeze and thaw cycles on stability of artelinic acid in human plasma samples was determined as follows: Spiked (40 and 800 ng/ml artelinic acid concentrations) pooled biological sample were aliquoted (1 ml) to screw top culture tubes and subjected to five thaw/freeze cycles. Each cycle, a duplicate set of thaw/freeze samples was generated.

Run the study with the following procedure:

a. Prepare high and low concentration samples labelled H-1, H-2 ... H-5, and L-1, L-2 ... L-5, in duplicate.

b. Store all samples until frozen at the specified temperature.

c. Repeatedly thaw and refreeze samples according to the following table. Thaw as if for sample preparation to room temperature. Let thawed samples stand at room temperature for 1 hour.

Cycle	Keep these samples in freezer	Thaw these samples
1	1	2, 3, 4, 5
2	1, 2	3, 4, 5
3	1, 2, 3	4, 5
4	1, 2, 3, 4	5
5	1, 2, 3, 4, 5	none

d. Following Cycle 5, take out all of the samples, thaw to room temperature, and assay the samples with a standard curve.

J. RESULTS

1. STANDARD CURVE

   Chromatograms for each point in representative standard curves for artelinic acid appear in Figure 1. Peak height ratios for these calibrators appear in Table 1.

2. RECOVERY

   Results for this evaluation appear in Table 2.

3. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 3 and 4.

4. STANDARD CURVE STUDY STATISTICS

   Mean, standard deviation, percent coefficient of variation and percent deviation from corresponding calibrator concentrations of the standard curve calibrators for this study appear in Table 5.

5. LOW POINT VALIDATION

   Take the 6 back calculated lowest standard calibrator concentrations that were obtained in the interday precision-accuracy study as the quantitation limit interday result. Results appear in Table 6.

6. STABILITY

   a. Freeze and Thaw

      Results appear in Table 7.

   b. Freezer Storage Stability

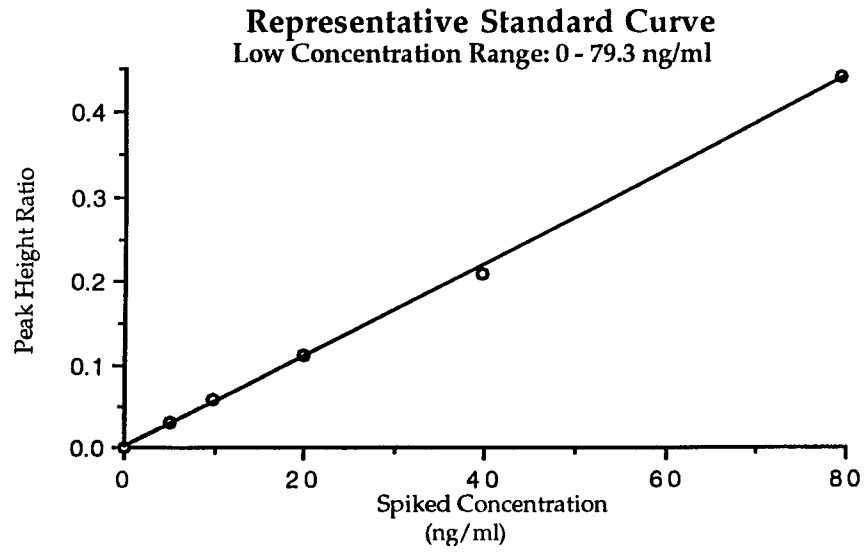
      Freezer storage stability data is presented in Table 8.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR ARTELINIC ACID HUMAN PLASMA ASSAY STUDY REPORT 20

SPIKED AMOUNT (ng)	STANDARD CURVE CONCENTRATION* (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
4.96	4.96	0.031	5.40[a]
9.92	9.92	0.059	10.5[a]
19.8	19.8	0.112	20.2[a]
39.7	39.7	0.209	37.8[a]
79.3	79.3	0.441	80.1[a]
159	159	0.910	158[b]
317	317	1.804	315[b]
635	635	3.779	661[b]
1270	1270	7.180	1260[b]

Regression equations:***
[a] $y = 0.00549x + 0.0013$, $r^2 = 0.9989$; (Low Range: 0 - 79.3 ng/ml)
[b] $y = 0.00570x + 0.0069$, $r^2 = 0.9994$; (High Range: 0 - 1270 ng/ml)

* When 1 ml of biological sample is used.
** Ratio of drug peak height to internal standard peak height.
*** Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level concentrations, two standard curves were constructed from the same set of standard curve data points.



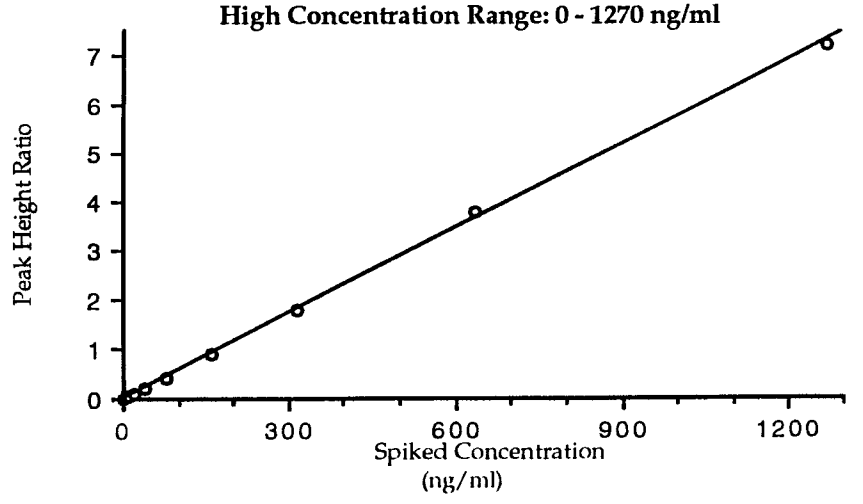


TABLE 2: ARTELINIC ACID MINIMUM QUANTITATION LIMIT

NH2 Method

Spiked Concentration	(9.49 ng/ml)	(9.5 ng/ml)	(4.75 ng/ml)
	Measured Concentrations (ng/ml)		
	Interday	Intraday	Interday
	9.03	12.1	5.20
	9.81	8.35	4.55
	9.03	9.72	5.20
	9.29	9.03	bc
	9.77	9.72	6.38
	9.82	11.8	4.19
Mean	9.46	10.1	5.10
Standard Deviation	0.386	1.51	0.835
Percent CV	4.09	14.9	16.4
Percent Error	-0.334	6.53	7.45

SAX Method

Spiked Concentration	(9.92 ng/ml)	(9.96 ng/ml)	(4.96 ng/ml)
	Measured Concentrations (ng/ml)		
	Interday	Intraday	Interday
	9.97	9.95	6.13
	bc	10.5	6.30
	8.75	9.77	5.09
	8.92	8.32	bc
	9.98	10.1	bc
	9.51	9.41	4.00
Mean	9.43	9.68	5.38
Standard Deviation	0.575	0.755	1.06
Percent CV	6.10	7.81	19.8
Percent Error	-4.98	-2.86	8.47

TABLE 3: RECOVERY OF ARTELINIC ACID FROM HUMAN PLASMA

SAMPLE ID	SPIKED CONCENTRATION (ng/ml)	PERCENT RECOVERY	PEAK HEIGHT RATIO	
			SOLVENT	PLASMA
SAX CARTRIDGE				
		Low Concentration Range		
1	19.9		0.122	0.113
2			0.123	0.107
3			0.124	0.076
Mean (± SD)		80.1	0.123 ± 0.001	0.098 ± 0.020
		Medium Concentration Range		
1	159		1.194	1.028
2			1.147	1.013
3			1.126	0.980
Mean (± SD)		87.1	1.156 ± 0.035	1.007 ± 0.025
		High Concentration Range		
1	319		2.192	2.012
2			2.222	1.898
3			2.311	2.041
Mean (± SD)		88.5	2.242 ± 0.062	1.984 ± 0.076
AVERAGE RECOVERY =		85.3		
NH2 CARTRIDGE				
		Low Concentration Range		
1			0.052	0.039
2			0.052	0.032
3			0.052	0.033
Mean (± SD)		67.4	0.052 ± 0.000	0.035 ± 0.004
		Medium Concentration Range		
1			0.427	0.332
2			0.431	0.318
3			0.441	0.340
Mean (± SD)		76.2	0.433 ± 0.007	0.330 ± 0.011
		High Concentration Range		
1			1.786	1.348
2			1.797	1.340
3			1.803	1.274
Mean (± SD)		73.6	1.795 ± 0.009	1.321 ± 0.041
AVERAGE RECOVERY =		72.4		

TABLE 4A: INTERDAY PRECISION OF ARTELINIC ACID HUMAN PLASMA ASSAY (NH2 METHOD)

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
	Measured Concentrations (ng/ml)									
9.50	9.79	9.62	9.79	8.85	10.1	9.59	9.62	0.42	4.36	1.30
38.0	37.9	39.5	37.9	33.9	35.9	38.2	37.2	1.99	5.35	-2.06
380	376	340	396	365	338	320	356	28.1	7.91	-6.36
760	757	842	793	756	741	736	771	40.2	5.21	1.43

TABLE 4B: INTRADAY PRECISION OF ARTELINIC ACID HUMAN PLASMA ASSAY (NH2 METHOD)

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
	Measured Concentrations (ng/ml)									
9.50	12.1	8.35	9.72	9.03	9.72	11.8	10.1	1.51	14.9	6.53
38.0	34.3	33.6	37.4	35.3	38.4	42.2	36.9	3.19	8.65	-2.98
380	369	344	393	359	349	382	366	19.1	5.21	-3.68
760	758	671	733	707	758	773	733	38.39	5.24	-3.51

*Measured concentrations are averages of two analyses.

TABLE 4C: INTERDAY PRECISION OF ARTELINIC ACID HUMAN PLASMA ASSAY (SAX METHOD)

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
	Measured Concentrations (ng/ml)									
9.96	9.15	9.64	9.33	9.10	11.4	9.43	9.68	0.867	8.97	-2.86
39.8	42.1	38.8	41.6	36.2	36.9	41.0	39.4	2.51	6.37	-0.921
398	401	398	397	420	414	419	408	10.7	2.62	2.55
797	813	790	754	839	804	810	802	28.3	3.53	0.586

TABLE 4D: INTRADAY PRECISION OF ARTELINIC ACID HUMAN PLASMA ASSAY (SAX METHOD)

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
	Measured Concentrations (ng/ml)									
9.96	9.95	10.5	9.77	8.32	10.1	9.41	9.68	0.755	7.81	-2.86
39.8	39.5	40.0	41.5	40.7	39.5	41.3	40.4	0.882	2.18	1.55
398	386	397	383	399	400	398	394	7.36	1.87	-1.05
797	789	780	783	767	772	806	783	13.8	1.76	-1.78

*Measured concentrations are averages of two analyses.

TABLE 5: PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS FOR ARTELINIC ACID HUMAN PLASMA ASSAY

Standard Curve Concentration (ng/ml)	n	Mean (ng/ml)	Standard Deviation (ng/ml)	Percent Coefficient of Variation	Percent Deviation
		NH2 METHOD			
4.75	6	5.08	0.750	14.8	6.84
9.49	7	9.40	0.388	4.13	-0.978
19.0	7	19.1	1.32	6.93	0.376
38.0	7	36.5	2.74	7.51	-3.95
75.9	7	76.6	1.48	1.93	0.960
152	7	151	6.23	4.13	-0.752
304	7	316	17.3	5.48	3.85
608	6	600	61.5	10.3	-1.29
1220	7	1217	26.9	2.21	-0.234
		SAX METHOD			
4.96	5	5.38	0.922	17.1	8.55
9.92	6	9.61	0.676	7.04	-3.18
19.8	7	19.8	1.30	6.58	0.144
39.7	7	38.3	1.55	4.06	-3.63
79.3	7	80.0	0.796	0.994	0.937
159	7	162	6.23	3.85	1.80
317	6	318	8.48	2.66	0.421
635	7	646	20.0	3.09	1.80
1270	7	1264	12.7	1.01	-0.450

TABLE 6: ACCURACY OF ARTELINIC ACID HUMAN PLASMA ASSAY (BLIND STUDY RESULTS) May 93

Sample Number	Spiked Level (ng/ml)	Measured Level (ng/ml)	Statistics (ng/ml)
1	0	*	Mean =
12		*	SD =
18		*	Percent CV =
20		*	Percent Bias =
2	23.54	21.6	Mean = 20.5
7		21.4	SD = 1.23
17		19.6	Percent CV = 6.00
19		19.2	Percent Bias = -13.1
3	41.3	36.3	Mean = 40.4
10		37.6	SD = 4.48
15		41.3	Percent CV = 11.1
21		46.3	Percent Bias = -2.24
4	124.0	111	Mean = 119
11		123	SD = 5.26
14		119	Percent CV = 4.44
24		121	Percent Bias = -4.44
5	200.7	196	Mean = 196
8		200	SD = 3.69
13		191	Percent CV = 1.88
22		196	Percent Bias = -2.47
6	401.4	406	Mean = 404
9		393	SD = 8.35
16		413	Percent CV = 2.07
23		402	Percent Bias = 0.523

#n = 3, unless a chromatogram is determined to be unacceptable.

TABLE 7: STABILITY OF ARTELINIC ACID IN HUMAN PLASMA

Artelinic Acid Concentration of Samples Stored at -20°C

	CONCENTRATION (ng/ml)			
Spiked Concentration:	9.50	38.0	380	760
TIME STORED				
0 days	bc	36.9	407	811
1 day	bc	38.8	388	737
2 days	bc	21.0	180	640
3 days	8.07	40.6	310	688
4 days	7.96	39.5	390	813
1 week	7.92	30.6	509	820
11 days	bc	46.1	225	814
2 week	13.8	40.5	383	813
22 days	11.4	50.5	414	868
1 month	11.1	38.7	371	765
2 months	10.5	39.4	379	776
3 months	9.68	32.9	295	682
6 months	10.4	42.4	392	717

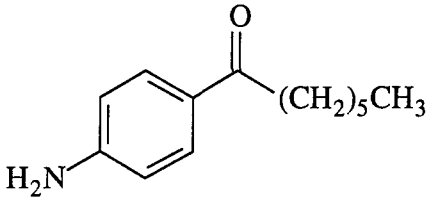
TABLE 8: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON ARTELINIC ACID SPIKED HUMAN PLASMA SAMPLES

	ARTELINIC ACID	
	Low Concentration	High Concentration
Spiked Concentration	(40.0 ng/ml)	(800 ng/ml)
Cycle		
1	35.4	770
2	40.4	731
3	33.9	676
4	32.0	728
5	38.2	735

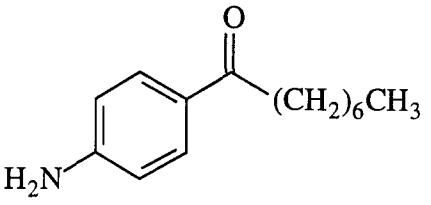
**Measured concentrations are averages of two analyses.
#Concentrations are mean (n = 2) results, except when chromatograms are unacceptable.

10/19/93

ANALYTICAL STANDARD OPERATING PROCEDURE (SOP) FOR AUTOMATED HPLC ASSAY FOR *p*-AMINOHEPTANOPHENONE IN DOG PLASMA



p-Aminoheptanophenone
(WR 269,410)



Internal Standard
p-Aminooctanophenone
(WR 258,948)

APPROVALS:

This Analytical Standard Operating Procedure is approved for use in

Study Number: _____

	INITIALS	DATE
Study Leader:	_____	_____
QA Officer:	_____	_____

INSTRUMENTS:

PUMP:	LC-600 Shimadzu Pump, or equivalent.
INJECTOR:	Waters Intelligent Sample Processor Model 710 B (WISP), or equivalent.
COLUMN:	Beckman ODS 5 µm, 25 cm X 4.6 mm, or equivalent.
DETECTOR:	Kratos Spectorflow 773, or equivalent.
INTEGRATOR:	Hewlett Packard Integrator 3392A, or equivalent.

CONDITIONS:

FLOW:	1.3 ml/min
INJECTION VOLUME:	50 - 80 µl
RUN TIME:	22 min (PAOP (Internal Standard): 16.5 min; PAHP: 10.2 min)
DETECTOR SETTINGS:	Wavelength: 316 nm Absorption Range: 0.006 aufs Rise Time 1.0 s
MOBILE PHASE:	Acetonitrile/Water (50:50, v/v) and 0.15% H_3PO_4

STANDARDS:

1. STOCK SOLUTIONS

   p-Aminoheptanophenone (WR 269,410)-(WRAIR, Washington, D.C.), bottle number BM 11565.

   10.22 mg /100 ml in methanol
   Conc. = 102 µg/ml

   p-Aminooctanophenone Internal Standard (WR 258,948) - (WRAIR, Washington, D.C.), bottle number BM 11207.

   10.5 mg /100 ml in methanol
   Conc. = 105 µg/ml

2. WORKING SOLUTIONS

   p-Aminoheptanophenone - Take 2.0 ml of 102 µg/ml stock and q.s. to 20 ml with methanol.

   Conc. = 10.2 µg/ml

   p-Aminoheptanophenone - Take 2.0 ml of 10.2 µg/ml working and q.s. to 20 ml with methanol.

   Conc. = 1.02 µg/ml

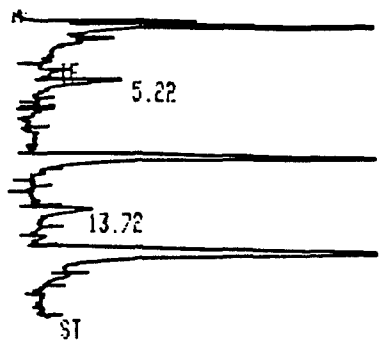
   p-Aminooctanophenone Internal Standard - Take 1.0 ml of 105 µg/ml stock and q.s. to 1000 ml with methanol.

   Conc. = 1.05 µg/ml

SAMPLE PREPARATION:

1. Pipet 0.5 ml of dog plasma samples into 16 X 125 culture tubes.
2. Spike standard curve samples with 00, 0, 2, 4, 8, or 15 µl of 1.02 µg/ml *p*-aminoheptanophenone working solution or 3, 6, 10, 20, or 40 µl of 10.2 µg/ml *p*-aminoheptanophenone working solution to make a standard curve. This procedure is equivalent to addition of 0, 0, 2.04, 4.08, 8.16, 15.3, 30.6, 61.2, 102, 204, and 408 ng of *p*-aminoheptanophenone , respectively, to each sample. Since 0.5 ml plasma clinical samples are assayed, these amounts correspond to 00, 0, 4.08, 8.16, 16.3, 30.6, 61.2, 122, 204, 408, and 816 ng/ml *p*-aminoheptanophenone concentrations. Vortex for 30 s.
3. Add 50 µl of 1.05 µg/ml *p*-aminooctanophenone internal standard solution. Vortex for 1 min.
4. Add 50 µl of 1N NaOH. Vortex 30 s.
5. Add 5 ml methyl *t*-butyl ether and cap tubes. Vortex 1 min. Centrifuge 10 min at 3000 *g*.
6. Freeze in dry ice/methanol bath. Transfer organic layer to silanized 13 X 100 culture tubes and evaporate to dryness.
7. Immediately reconstitute residue with 200 µl of mobile phase. Vortex for 1 min.
8. Transfer to WISP inserts and inject 50 - 80 µl onto column.

COMMENTS: Use reverse side if necessary.



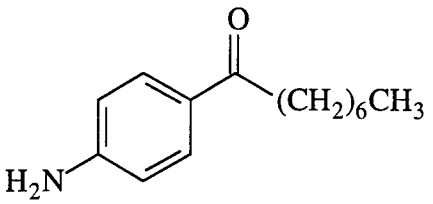

```
                        RUN #   29              JUN/11/93  03:22:27

                        ISTD
                          RT        HEIGHT TYPE  CAL#        AMOUNT
                         5.22         2398   BB                0.000
                        10.21        11677   BB    1R          0.983
LIST: METH @            13.72         1578   BB                0.000
                        16.51        11882   BB    2&          1.000
RUN PRMTRS
ZERO    =  30
ATT 2↑  =  1            TOTAL HGHT=       27535
CHT SP  =  0.2            ISTD AMT= 1.0000E+00
PK WD   =  0.16         MUL FACTOR= 1.0000E+00
THRSH   =  2
AR REJ  =  0

RPRT OPTNS
  2. RF UNC PKS=       0.0000E+00
  3. MUL FACTOR=       1.0000E+00
  4. PK HEIGHT MODE      YES
  5. EXTEND RT            NO
  6. RPRT UNC PKS        YES

TIME TBL
   0.00 INTG # =  9
   4.00 INTG # =  -9
   4.00 INTG # =  2
  13.00 STOP

CALIB TBL
ISTD                    CALIB RUNS  1
REF % RTW=  10.00       % RTW=  10.00

CAL #      RT          AMT         AMT/HEIGHT
  1R     11.15     1.0000E+00    1.0000E+00
  2&     18.10     1.0000E+00    1.0000E+00
```

WRITTEN BY: Mila Hanz DATE: 10/11/93

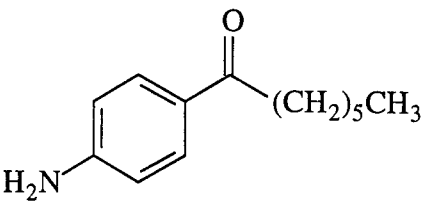
NEW SOP ✓ REVISION_____

10/19/93

ANALYTICAL STANDARD OPERATING PROCEDURE (SOP) FOR AUTOMATED HPLC ASSAY FOR *p*-AMINOOCTANOPHENONE IN DOG PLASMA



p-Aminooctanophenone
(WR 258,948)



Internal Standard
p-Aminoheptanophenone
(WR 269,410)

APPROVALS:

This Analytical Standard Operating Procedure is approved for use in

Study Number: _____

	INITIALS	DATE
Study Leader:	_____	_____
QA Officer:	_____	_____

INSTRUMENTS:

PUMP:	LC-600 Shimadzu Pump, or equivalent.
INJECTOR:	Waters Intelligent Sample Processor Model 710 B (WISP), or equivalent.
COLUMN:	Beckman ODS 5 µm, 25 cm X 4.6 mm, or equivalent.
DETECTOR:	Kratos Spectorflow 773, or equivalent.
INTEGRATOR:	Hewlett Packard Integrator 3392A, or equivalent.

CONDITIONS:

FLOW:	1.3 ml/min
INJECTION VOLUME:	50 - 80 µl
RUN TIME:	22 min (PAOP: 18.0 min; PAHP (Internal Standard): 11.2 min)
DETECTOR SETTINGS:	Wavelength: 316 nm Absorption Range: 0.006 aufs Rise Time 1.0 s
MOBILE PHASE:	Acetonitrile/Water (50:50, v/v) and 0.15% H_3PO_4

STANDARDS:

1. STOCK SOLUTIONS

p-Aminooctanophenone (WR 258,948) - (WRAIR, Washington, D.C.), bottle number BM 11207.

10.4 mg /100 ml in methanol
Conc. = 104 µg/ml

p-Aminoheptanophenone Internal Standard (WR 269,410) - (WRAIR, Washington, D.C.), bottle number BM 11565.

9.69 mg /100 ml in methanol
Conc. = 96.9 µg/ml

1. WORKING SOLUTIONS

p-Aminooctanophenone - Take 2.0 ml of 104 µg/ml stock and q.s. to 20 ml with methanol.

Conc. = 10.4 µg/ml

p-Aminooctanophenone - Take 2.0 ml of 10.4 µg/ml working and q.s. to 20 ml with methanol.

Conc. = 1.04 µg/ml

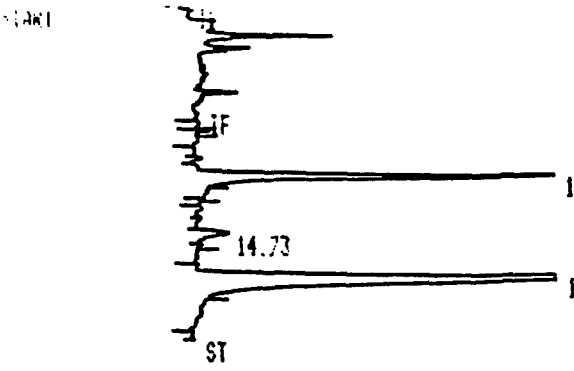
p-Aminoheptanophenone Internal Standard - Take 1.0 ml of 96.9 µg/ml stock solution and q.s. to 100 ml with methanol.

Conc. = 0.969 µg/ml

SAMPLE PREPARATION:

1. Pipet 0.5 ml of dog plasma samples into 16 X 125 culture tubes.
2. Spike standard curve samples with 00, 0, 2, 4, 8, or 15 µl of 1.04 µg/ml *p*-aminooctanophenone working solution or 3, 6, 10, 20, or 40 µl of 10.4 µg/ml *p*-aminooctanophenone working solution to make a standard curve. This procedure is equivalent to addition of 0, 0, 2.08, 4.16, 8.32, 15.6, 31.2, 62.4, 104, 208, and 416 ng of *p*-aminooctanophenone , respectively, to each sample. Since 0.5 ml plasma clinical samples are assayed, these amounts correspond to 00, 0, 4.16, 8.32, 16.6, 31.2, 62.4, 125, 208, 416, and 832 ng/ml *p*-aminooctanophenone concentrations. Vortex for 30 s.
3. Add 30 µl of 0.969 µg/ml *p*-aminoheptanophenone internal standard solution. Vortex for 1 min.
4. Add 50 µl of 1N NaOH. Vortex 30 s.
5. Add 5 ml methyl *t*-butyl ether and cap tubes. Vortex 1 min. Centrifuge 10 min at 3000 *g*.
6. Freeze in dry ice/methanol bath. Transfer organic layer to silanized 13 X 100 culture tubes and evaporate to dryness.
7. Immediately reconstitute residue with 200 µl of mobile phase. Vortex for 1 min.
8. Transfer to WISP inserts and inject 50 - 80 µl onto column.

COMMENTS: Use reverse side if necessary.

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                        WORKFILE ID: C
                        WORKFILE NAME:

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ATT 2↑ =  1              17.69       28117   PB    2&        1.000
CHT SP =  0.2
PK WD  =  0.16
THRSH  =  2             TOTAL HGHT=      42167
AR REJ =  0             ISTD AMT= 1.0000E+00
                        SAMPLE AMT=
RPRT OPTNS              MUL FACTOR= 1.0000E+00
  2. RF UNC PKS=      1.0000E+00
  3. MUL FACTOR=      1.0000E+00
  4. PK HEIGHT MODE     YES
  5. EXTEND RT           NO
  6. RPRT UNC PKS       YES

TIME TBL
   0.00 INTG # =  9
   4.00 INTG # =  2
   9.50 INTG # =  -9
  25.00 STOP

CALIB TBL
ISTD                    CALIB RUNS  1
REF % RTW=  10.00       % RTW=  10.00

CAL #      RT          AMT          AMT/HEIGHT
  1S     11.11     7.1881E+04     1.0000E+00
  2      18.02     3.6478E+04     1.0000E+00
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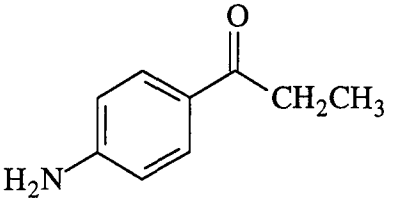
WRITTEN BY: Julia Hawn DATE: 10/22/93

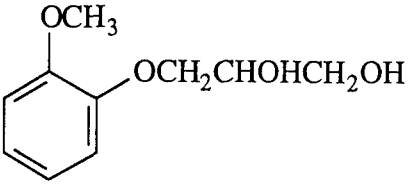
NEW SOP ✓ REVISION _____

10/19/93

ANALYTICAL STANDARD OPERATING PROCEDURE (SOP) FOR AUTOMATED HPLC ASSAY FOR *p*-AMINOPROPIOPHENONE IN DOG PLASMA



p-Aminopropiophenone
(WR 000,302)



Guaifenesin
Internal Standard

APPROVALS:

This Analytical Standard Operating Procedure is approved for use in

Study Number: _____

	INITIALS	DATE
Study Leader:	_____	_____
QA Officer:	_____	_____

INSTRUMENTS:

PUMP:	LC-600 Shimadzu Pump, or equivalent.
INJECTOR:	Waters Intelligent Sample Processor Model 710 B (WISP), or equivalent.
COLUMN:	Beckman ODS 5 µm, 25 cm X 4.6 mm, or equivalent.
DETECTOR:	Kratos Spectorflow 773, or equivalent.
INTEGRATOR:	Hewlett Packard Integrator 3392A, or equivalent.

CONDITIONS:

FLOW:	1.0 ml/min
INJECTION VOLUME:	40 - 80 µl
RUN TIME:	14 min (PAPP: 10.7 min; Guaifenesin (Internal Standard): 8.5 min)
DETECTOR SETTINGS:	Wavelength: 316 nm Absorption Range: 0.006 aufs Rise Time 1.0 s
MOBILE PHASE:	Acetonitrile/Water (20:80, v/v) and 0.15% H_3PO_4

STANDARDS:

1. STOCK SOLUTIONS

p-Aminopropiophenone (WR 000,302) - (WRAIR, Washington, D.C.), bottle number BM 11449.

10.10 mg /100 ml in methanol
Conc. = 101 µg/ml

Guaifenecin (Internal standard) - (K & K Labs)

9.94 mg /100 ml in methanol
Conc. = 99.4 µg/ml

1. WORKING SOLUTIONS

p-Aminopropiophenone - Take 2.0 ml of 101 µg/ml stock solution and q.s. to 20 ml with methanol.

Conc. = 10.1 µg/ml

p-Aminopropiophenone - Take 2.0 ml of 10.1 µg/ml working solution and q.s. to 20 ml with methanol.

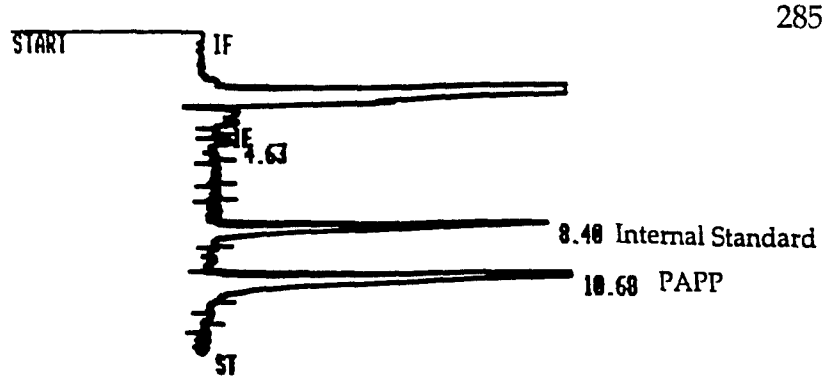
Conc. = 1.01 µg/ml

Guaifenecin (Internal standard) - Same as stock solution.
Conc. = 99.4 µg/ml

SAMPLE PREPARATION:

1. Pipet 0.5 ml of dog plasma samples into 16 X 125 culture tubes.
2. Spike standard curve samples with 00, 0, 2, 4, 8, or 15 µl of 1.01 µg/ml *p*-aminopropiophenone working solution or 3, 6, 10, 20, or 40 µl of 10.1 µg/ml *p*-aminopropiophenone working solution to make a standard curve. This procedure is equivalent to addition of 0, 0, 2.02, 4.04, 8.08, 15.2, 30.3, 60.6, 101, 202, and 404 ng of *p*-aminopropiophenone, respectively, to each sample. Since 0.5 ml plasma clinical samples are assayed, these amounts correspond to 00, 0, 4.04, 8.08, 16.2, 30.3, 60.6, 121, 202, 404, and 808 ng/ml *p*-aminopropiophenone concentrations. Vortex for 30 s.
3. Add 30 µl of 99.4 µg/ml guaifenecin internal standard solution. Vortex for 1 min.
4. Add 50 µl of 1N NaOH. Vortex 30 s.
5. Add 5 ml methyl *t*-butyl ether and cap tubes. Vortex 1 min, twice. Centrifuge 10 min at 3000 *g*.
6. Freeze in dry ice/methanol bath. Transfer organic layer to silanized 13 X 100 culture tubes and evaporate to dryness.
7. Immediately reconstitute residue with 200 µl of mobile phase. Vortex for 1 min.
8. Transfer to WISP inserts and inject 40 - 80 µl onto column.

COMMENTS: Use reverse side if necessary.

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PK WD  =  0.16
THRSH  =  2
AR REJ =  0                     TOTAL HGHT=      27773
                                ISTD AMT= 1.0000E+00
                                SAMPLE AMT=
                                MUL FACTOR= 1.0000E+00

RPRT OPTNS
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  3. MUL FACTOR=       1.0000E+00
  4. PK HEIGHT MODE      YES
  5. EXTEND RT            NO
  6. RPRT UNC PKS        YES

TIME TBL
    0.00 INTG # =  9
    4.00 INTG # =  -9
    4.00 INTG # =  2
   14.00 STOP

CALIB TBL
ISTD                    CALIB RUNS  1
REF % RTW=  10.00       % RTW=  10.00

CAL #     RT          AMT          AMT/HEIGHT
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  2&      8.51    1.0000E+00     1.0000E+00
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WRITTEN BY: Mila Starr DATE: 10/22/93

NEW SOP ✓ REVISION_____

I. LABORATORY METHODOLOGY FOR WR 6026, WR 211,789 AND WR 254,421 (AS FREE BASES) URINE ASSAY, STUDY REPORT 22

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 712 (Waters Associates, Milford, MA), or equivalent.

2. Altex Model 100A Solvent Delivery Module (Beckman Instruments, Inc., Berkeley, CA), or equivalent.

3. Kratos Spectroflow 783 UV Detector (Kratos Analytical Instruments, Ramsey, NJ), or equivalent.

4. Hewlett-Packard Reporting Integrator #3390A (Hewlett-Packard Co., Santa Clara, CA), or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.

2. All chemicals are reagent grade.

3. WR 6026•2HCl - bottle no. BK 01845 (WRAIR, Washington, DC).

4. WR 211,789•2HCl•1/2H_2O - bottle no. BK 50713 (WRAIR, Washington, DC).

5. WR 254,421•2HCl - bottle no. BK 18756 (WRAIR, Washington, DC).

6. Verapamil (Internal Standard) - (USP Reference Lot # F-1)

7. NaOH (Mallinckrodt, Paris, KY).

8. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

9. Methyl-*t*-butyl ether (Baxter, Burdick & Jackson, Muskegan, MI).

10. Methanol (Fisher Scientific, Fairlawn, NJ).

11. Acetonitrile (Fisher Scientific, Fairlawn, NJ).

12. Phosphoric acid (Fisher Scientific, Fair Lawn, NJ).

*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

C. ASSAY CONDITIONS

1. DETECTOR

   Settings

   Wavelength; 350 nm
   Sensitivity; 0.005 aufs
   Rise Time; 1 s

   Lamp

   ABI Analytical, Inc. (Ramsey, NJ).

2. COLUMN

   Axxiom Si, 5 µm particle size, 4.6 x 250 mm - (Richard Scientific, Novato, CA).

3. MOBILE PHASE

   CH_3CN/0.0075% H_3PO_4 (80:20, v/v) with final apparent pH of 6.9.

4. FLOW RATE

   1.0 ml/min

5. STOCK SOLUTIONS - Store at 4°C, protect from light, and check for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (discard solutions if a more than 10% change in the ratio is observed). In any case, discard solutions within 6 months.

   a. Precision solutions.

Preparation date: 2/9/93

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Free Base Conc. (mg/ml)
WR 6026	3.140	0.8249*	10	50% meOH	0.259
WR 211,789	2.138	0.7938**	10	50% meOH	0.170
WR 254,421	1.821	0.8314***	10	50% meOH	0.151
Verapamil Internal Std.	6.873	1	6.873	50% meOH	1

*= Molecular weights of WR 6026 free base/WR 6026 •2HCl
**= Molecular weights of WR 211,789 free base/WR 211,789•2HCl•1/2H_2O
***= Molecular weights of WR 254,421free base/WR 254,421•2HCl

b. Stability and blind sample analysis solutions. (WR 6026 and WR 211,789 solutions same as precision solutions.)

Preparation date: 3/26/93

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Free Base Conc. (µg/ml)
WR 254,421	14.346	0.8314		50% meOH	543

6. WORKING SOLUTIONS - (Store solutions at 4°C, protect from light, and discard if deterioration is observed in the stock solutions). In any case, solutions are discarded within 6 months.

a. Combine and dilute stock precision solutions.

Preparation date: 2/9/93

Solution Type	Conc. Diluted (mg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
WR 6026	0.259	1:100	50% meOH	2.59
WR 211,789	0.170	1.5:100	50% meOH	2.55
WR 254,421	0.151	15:100	50% meOH	22.7

b. Combine and dilute stability and blind sample analysis solutions.

Preparation date: 3/26/93

Solution Type	Conc. Diluted (mg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
WR 6026	0.259	4:18.4	50% meOH	56.3
WR 211,789	0.170	6:18.4	50% meOH	55.4
WR 254,421	1.19	8.4:18.4	50% meOH	543

c. Stability and blind sample analysis solutions.

Preparation date: 3/26/93

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
WR 6026	56.3	4.6:10	50% meOH	25.9
	25.9	1:10	50% meOH	2.59
WR 211,789	55.4	4.6:10	50% meOH	25.5
	25.5	1:10	50% meOH	2.55
WR 254,421	543	4.6:10	50% meOH	250
	250	1:10	50% meOH	25.0

d. Verapamil (Internal Standard).

Preparation date: 2/9/93

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
Internal Std	1000	1:10	50% meOH	100
Working IS	100	1:2	50% meOH	50

7. RETENTION TIMES (subject to change depending on temperature and column performance). Approximate run time: 23 min.

   a. Verapamil (Internal Standard) - 12.4 min
   b. WR 211,789 (free base) - 14.3 min
   c. WR 6026 (free base) - 15.3 min
   d. WR 254,421 (free base) - 18.2 min

8. QUANTITATION

   By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by nonweighted linear regression.

9. MINIMUM DETECTION LIMIT OF METHOD FOR PRECISION ANALYSIS

   The minimum detection limits, 5.17 ng/ml WR 6026, 5.09 ng/ml WR 211,789, and 45.4 ng/ml WR 254,421 (as free bases) in urine, was determined as the WR 6026, WR 211,789, and WR 254,421 (as free bases) concentrations at which the signal to noise ratio was at least 3 to 1.

10. INJECTION VOLUME

    20 to 160 µl

11. SAMPLE VOLUME MEASUREMENT

    Pipetters used for sample volume measurement were Rainin, Gilson Pipettman and/or Eppendorf. See SOP 3-4.1 for calibration procedure.

12. BLANK URINE

    Spiked urine samples were made with interference-free urine obtained from UCSF Department of Pharmacy staff volunteers.

13. WISP OPERATING TEMPERATURE

    Room Temperature

D. SAMPLE STORAGE

Urine samples were kept frozen, if required at -70°C before analysis and thawed at room temperature for preparation and analysis.

E. SAMPLE PREPARATION

1. Pipette 0.5 ml urine into glass culture tube.

2. Spike precision standard curve samples with 00, 0, 1, 2, 3, 5, 10, 20, 40, or 80 µl of WR 6026, WR 211,789, and WR 254,421 standard curve working solution mixture (conc. 2.59 µg/ml of WR 6026, 2.55 µg/ml of WR 211,789 and 22.7 µg/ml of WR 254,421 as free bases) to make standard curves. This procedure is equivalent to addition of 00, 0, 2.59, 5.17, 7.76, 12.9, 25.9, 51.7, 103, or 207 ng of WR 6026, and 00, 0, 2.55, 5.09, 7.64, 12.7, 25.5, 50.9, 102, or 204 ng of WR 211,789 and 00, 0, 22.7, 45.4, 68.1, 114, 227, 454, 908, or 1820 ng of WR 254,421 to each sample. Since 0.5 ml urine clinical samples are assayed, these amounts correspond to 00, 0, 5.17, 10.3, 15.5, 25.9, 51.7, 103, 207, and 414 ng/ml WR 6026, 00, 0, 5.09, 10.2, 15.3, 25.5, 50.9, 102, 204, and 407 ng/ml WR 211,789 and 00, 0, 45.4, 90.8, 136, 227, 454, 908, 1820, and 3630 ng/ml WR 254,421 concentrations. Vortex 10 s.

3. Add 100 µl of internal standard (50 µg/ml verapamil) working solution. Vortex 10 s.

4. Add 100 µl of 1 N NaOH. Vortex 10 s.

5. Add 5 ml methyl *t*-butyl ether, cap and vortex 1 min.

6. Centrifuge 10 min at 3000 *g*.

7. Freeze in dry ice/methanol and transfer organic layer to a 13x100 mm culture tube.

8. Evaporate to dryness under nitrogen.

9. Reconstitute in 200 µl of mobile phase, vortex 1 min and inject 20-160 µl onto HPLC column.

F. QUALITY CONTROL

1. Content and frequency of blanks

*00 = Sample without drug and without internal standard.
**0 = Sample without drug and with internal standard.

No special blank was used except for the standard curve blank.

2. Pipette Calibration

   See SOP 3-4.1.

3. Balance Calibration

   See SOP 3-19.2.

G. RECOVERY

Assay recovery was assessed at three different concentrations by comparing the WR 6026, WR 211,789, and WR 254,421 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in urine. Urine and reference samples were spiked with corresponding amounts of WR 6026, WR 211,789, and WR 254,421 (as free bases). Each urine sample was prepared as described in "Sample Preparation" (Section E), except internal standard was added after the evaporation (step 8). The reference samples were spiked with WR 6026, WR 211,789, and WR 254,421 (as free bases) and with internal standard, but were not extracted and not evaporated.

H. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 0.5 ml urine specimens with WR 6026, WR 211,789, and WR 254,421 (as free bases) working solution as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Precision Samples

WR 6026 (as free base)

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	2.59	0.5	10.4
Low	5	2.59	0.5	25.9
Med.	30	2.59	0.5	155
Hi	50	2.59	0.5	259

WR 211,789 (as free base)

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	2.55	0.5	10.2
Low	5	2.55	0.5	25.5
Med.	30	2.55	0.5	153
Hi	50	2.55	0.5	255

WR 254,421 (as free base)

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	22.7	0.5	90.8
Low	5	22.7	0.5	227
Med.	30	22.7	0.5	1360
Hi	50	22.7	0.5	2270

I. GENERATION OF STABILITY SAMPLES

Long term stability samples were generated by spiking rat plasma samples as shown below.

WR 6026 (as free base)

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	2.59	0.5	10.4
Low	5	2.59	0.5	25.9
Med.	3	25.9	0.5	155
Hi	5	25.9	0.5	259

WR 211,789 (as free base)

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	2.55	0.5	10.2
Low	5	2.55	0.5	25.5
Med.	3	25.5	0.5	153
Hi	5	25.5	0.5	255

WR 254,421 (as free base)

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	25.0	0.5	100
Low	5	25.0	0.5	250
Med.	3	250	0.5	1500
Hi	5	250	0.5	2500

Autosampler stability samples were generated by spiking 0.5 ml human urine specimens with WR 6026, WR 211,789, and WR 254,421 working solution as shown above for long term stability sample.

J. RESULTS

1. STANDARD CURVE

Chromatograms for each point in a representative standard curve for WR 6026, WR 211,789, and WR 254,421 (as free bases) appear

in Figure 3. Peak height ratios for these calibrators appear in Tables 1A-C.

2. LOW POINT VALIDATION

   Take the 6 back calculated lowest standard calibrator concentrations that were obtained in the interday precision-accuracy study as the quantitation limit interday result. Results appear in Table 2.

3. RECOVERY

   Results for this evaluation appear in Table 3.

4. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Tables 4-6.

5. BLIND SAMPLE ANALYSIS

   Results for this evaluation appear in Tables 7A-C.

6. STABILITY

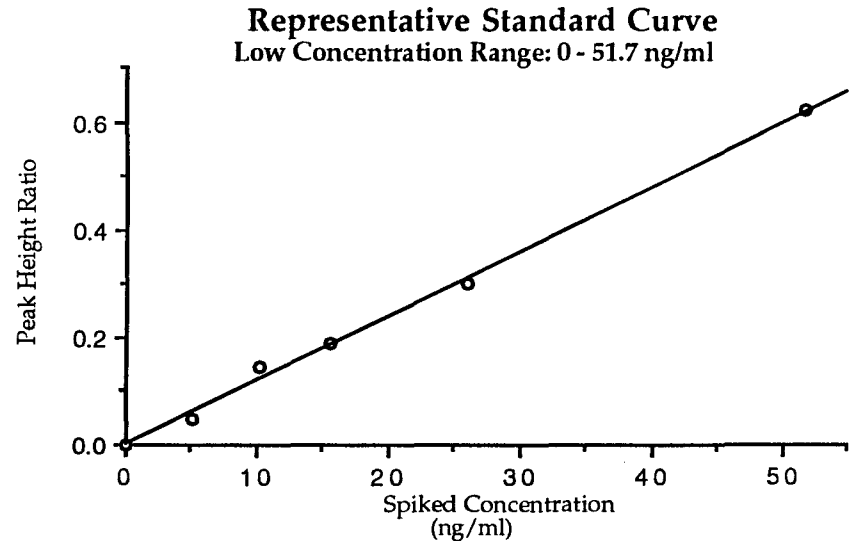
   Results for long term stability appear in Table 8. Results for autosampler stability appear in Table 9.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR WR 6026 HUMAN URINE ASSAY STUDY REPORT 22

SPIKED AMOUNT (ng)	STANDARD CURVE CONCENTRATION* (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
2.59	5.17	0.050	3.99[a]
5.17	10.3	0.148	12.2[a]
7.76	15.5	0.190	15.7[a]
12.9	25.9	0.301	25.0[a]
25.9	51.7	0.620	51.8[a]
51.7	103	1.358	126[b]
103	207	2.060	194[b]
207	414	4.357	414[b]

Regression equations:***
[a] $y = 0.01192x + 0.0024$, $r^2 = 0.9967$; (Low Range: 0 - 51.7 ng/ml)
[b] $y = 0.01040x + 0.0476$, $r^2 = 0.9951$; (High Range: 0 - 414 ng/ml)

* When 0.5 ml of biological sample is used.
** Ratio of drug peak height to internal standard peak height.
*** Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level concentrations, two standard curves were constructed from the same set of standard curve data points.



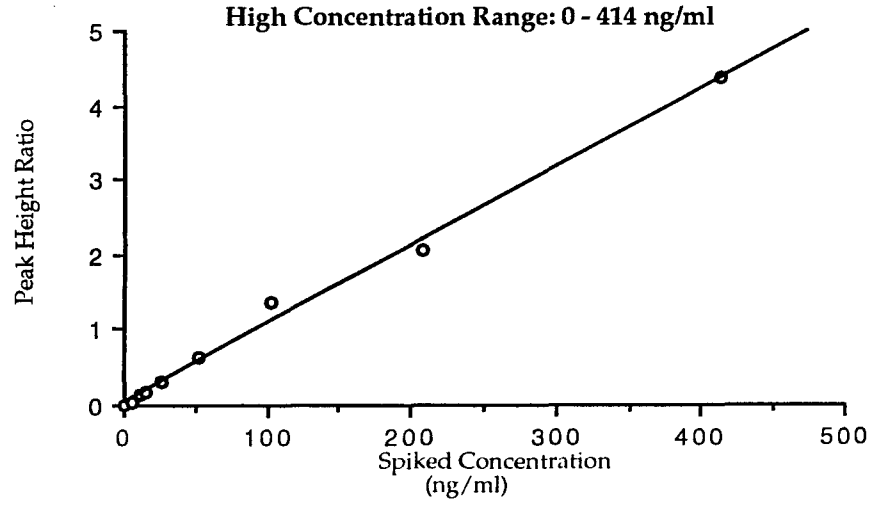
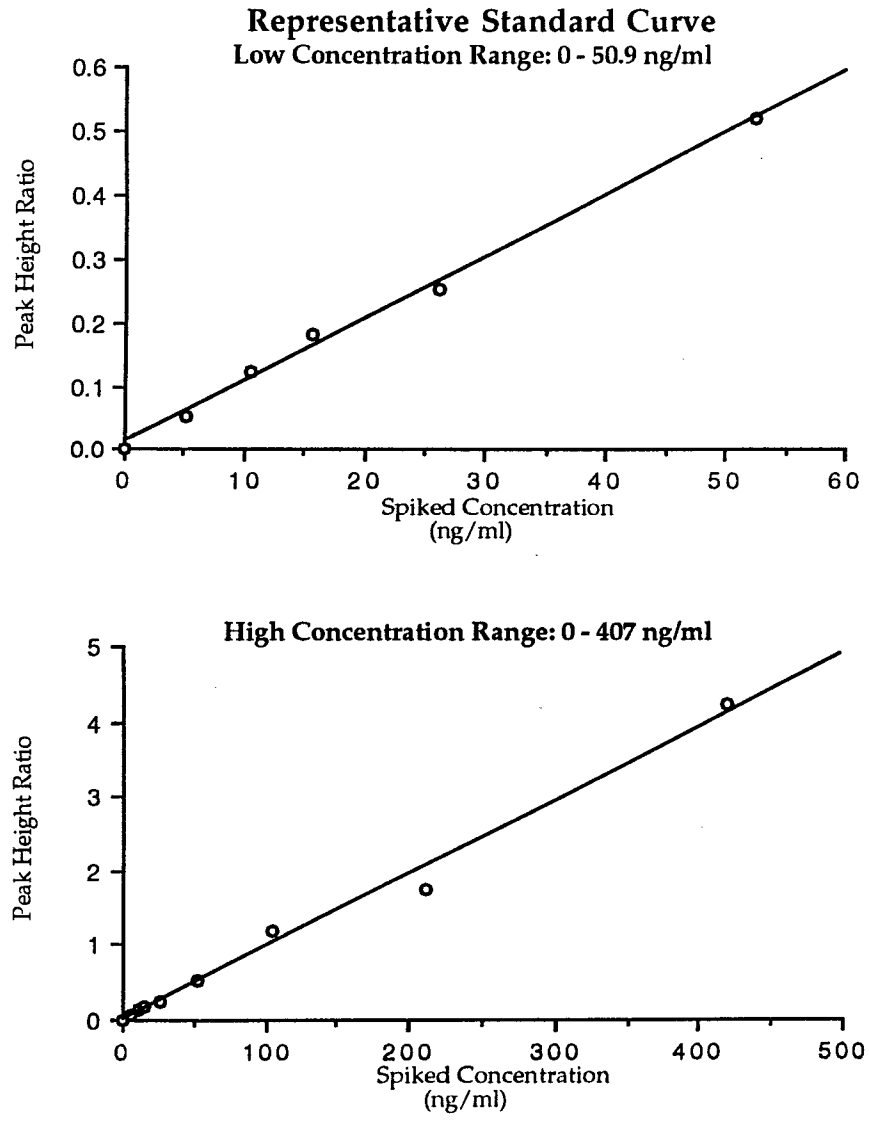


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR WR 211,789 HUMAN URINE ASSAY STUDY REPORT 22

SPIKED AMOUNT (ng)	STANDARD CURVE CONCENTRATION* (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
2.55	5.09	0.056	4.48[a]
5.09	10.2	0.124	11.3[a]
7.64	15.3	0.185	17.4[a]
12.7	25.5	0.253	24.2[a]
25.5	50.9	0.519	50.8[a]
50.9	102	1.185	117[b]
102	204	1.753	173[b]
204	407	4.224	418[b]

Regression equations:***
[a] $y = 0.01000x + 0.0112$, $r^2 = 0.9948$; (Low Range: 0 - 50.9 ng/ml)
[b] $y = 0.01009x + 0.,0028$ $r^2 = 0.9912$; (High Range: 0 - 407 ng/ml)



* When 0.5 ml of biological sample is used.
** Ratio of drug peak height to internal standard peak height.
*** Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level concentrations, two standard curves were constructed from the same set of standard curve data points.

TABLE 1C: REPRESENTATIVE STANDARD CURVE FOR WR 254,421 HUMAN URINE ASSAY STUDY REPORT 22

SPIKED AMOUNT (ng)	STANDARD CURVE CONCENTRATION* (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	0	-
22.7	45.4	0.497	46.1[a]
45.4	90.8	1.022	98.0[a]
68.1	136	1.458	141[a]
114	227	2.189	213[a]
227	454	4.664	458[a]
454	908	9.868	1070[b]
908	1820	15.346	1680[b]
1820	3630	32.938	3650[b]

Regression equations:***
[a] $y = 0.01012x + 0.0306$, $r^2 = 0.9979$; (Low Range: 0 - 454 ng/ml)
[b] $y = 0.00893x + 0.2975$, $r^2 = 0.9958$; (High Range: 0 - 3630 ng/ml)

* When 0.5 ml of biological sample is used.
** Ratio of drug peak height to internal standard peak height.
*** Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level concentrations, two standard curves were constructed from the same set of standard curve data points.

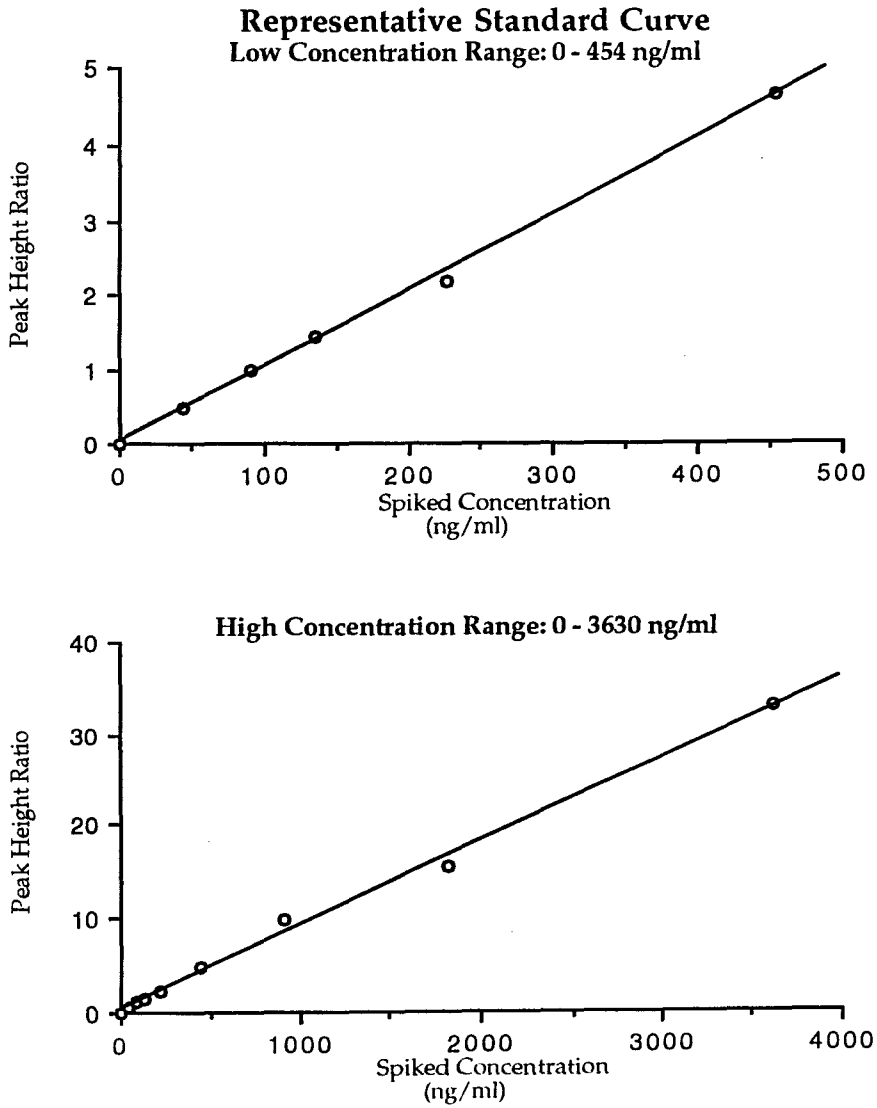


TABLE 2: WR 6026, WR 211,789, AND WR 254,421 (AS FREE BASES) MINIMUM QUANTITATION LIMITS

INTERDAY

Spiked Concentration	WR 6026 (5.17 ng/ml)	WR 211,789 (5.09 ng/ml)	WR 254,421 (45.4 ng/ml)
	Measured Concentrations (ng/ml)		
	bc	6.51	53.3
	6.10	4.76	46.8
	5.60	5.88	44.9
	5.39	5.50	45.3
	3.99	4.48	46.1
	5.40	4.80	42.4
Mean	5.30	5.32	46.1
Standard Deviation	0.785	0.78	3.47
Percent CV	14.8	14.7	7.51
Percent Error	2.44	4.55	1.60

TABLE 3: RECOVERIES OF WR 6026, WR 211,789 AND WR 254,421 (AS FREE BASES) FROM HUMAN URINE

SAMPLE ID	SPIKED CONCENTRATION Range (ng/ml)	PEAK HEIGHT RATIO REFERENCE	PEAK HEIGHT RATIO URINE	MEAN PERCENT RECOVERY
WR 6026				
1	Low	0.299	0.291	91.4
2		0.353	0.284	
3		0.340	0.335	
4		0.339	0.306	
Mean (± SD)		0.333 ± 0.023	0.304 ± 0.023	
1	Medium	1.254	1.380	100
2		1.519	1.393	
3		1.427	1.416	
4		1.381	1.394	
Mean (± SD)		1.395 ± 0.110	1.396 ± 0.015	
1	High	2.153	2.201	100
2		2.205	2.248	
3		2.204	2.261	
4		2.346	2.215	
Mean (± SD)		2.227 ± 0.083	2.231 ± 0.028	
AVERAGE (MEAN PERCENT RECOVERY) =				97.2

TABLE 3: RECOVERIES OF WR 6026, WR 211,789 AND WR 254,421 (AS FREE BASES) FROM HUMAN URINE (CONTINUED)

SAMPLE ID	SPIKED CONCENTRATION Range (ng/ml)	PEAK HEIGHT RATIO REFERENCE	PEAK HEIGHT RATIO URINE	MEAN PERCENT RECOVERY
WR 211,789				
1	Low	0.316	0.290	86.5
2		0.334	0.255	
3		0.333	0.320	
4		0.344	0.283	
Mean (± SD)		0.332 ± 0.012	0.287 ± 0.027	
1	Medium	1.226	1.315	96.0
2		1.519	1.320	
3		1.421	1.319	
4		1.339	1.330	
Mean (± SD)		1.376 ± 0.124	1.321 ± 0.006	
1	High	2.129	2.011	96.0
2		2.158	2.115	
3		2.156	2.174	
4		2.310	2.100	
Mean (± SD)		2.188 ± 0.082	2.100 ± 0.067	
AVERAGE (MEAN PERCENT RECOVERY) =				92.8

TABLE 3: RECOVERIES OF WR 6026, WR 211,789 AND WR 254,421 (AS FREE BASES) FROM HUMAN URINE (CONTINUED)

SAMPLE ID	SPIKED CONCENTRATION Range (ng/ml)	PEAK HEIGHT RATIO REFERENCE	PEAK HEIGHT RATIO URINE	MEAN PERCENT RECOVERY
WR 254,421				
1	Low	2.422	2.366	91.8
2		2.561	2.212	
3		2.711	2.512	
4		2.668	2.421	
Mean (± SD)		2.591 ± 0.129	2.378 ± 0.126	
1	Medium	10.126	9.939	94.6
2		10.942	10.282	
3		11.223	10.259	
4		10.896	10.388	
Mean (± SD)		10.797 ± 0.470	10.217 ± 0.194	
1	High	16.446	16.116	96.2
2		17.711	17.015	
3		17.265	16.684	
4		17.428	16.43	
Mean (± SD)		17.213 ± 0.543	16.561 ± 0.381	
AVERAGE (MEAN PERCENT RECOVERY) =				94.2

TABLE 4A: INTERDAY PRECISION OF WR 6026 HUMAN URINE ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6 Measured Concentrations** (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
10.4	11.2	11.7	12.1	11.5	9.74	10.8	11.2	0.83	7.42	7.44
25.9	23.0	25.8	23.2	24.3	25.3	22.8	24.1	1.27	5.28	-7.08
155	143	151	138	138	144	150	144	5.62	3.90	-7.10
259	255	275	220	257	248	245	250	18.0	7.22	-3.47

TABLE 4B: INTRADAY PRECISION OF WR 6026 HUMAN URINE ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6 Measured Concentrations (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
10.4	11.9	10.9	7.82	13.2	bc	6.37	10.0	2.85	28.4	-3.48
25.9	24.5	23.5	bc	24.2	25.3	26.2	24.7	1.04	4.21	-4.48
155	152	145	142	145	158	151	149	5.91	3.97	-3.98
259	237	232	239	236	237	258	240	9.20	3.83	-7.40

** Measured concentrations are averages of two analyses.

bc = bad chromatogram.

TABLE 5A: INTERDAY PRECISION OF WR 211789 HUMAN URINE ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6 Measured Concentrations** (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
10.2	9.20	11.2	11.1	11.9	10.2	11.4	10.8	0.97	8.98	6.21
25.5	21.5	25.9	22.7	21.8	27.2	22.5	23.6	2.36	10.0	-7.45
153	141	145	134	135	145	148	141	5.75	4.07	-7.63
255	255	270	215	253	253	240	248	18.6	7.52	-2.88

TABLE 5B: INTRADAY PRECISION OF WR 211789 HUMAN URINE ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6 Measured Concentrations (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
10.2	12.0	12.4	10.9	7.00	8.89	7.66	9.81	2.28	23.3	-3.84
25.5	22.3	25.5	20.8	24.7	27.1	19.1	23.3	3.04	13.1	-8.82
153	150	146	143	155	136	137	145	7.40	5.12	-5.56
255	241	233	227	228	218	255	234	12.9	5.52	-8.37

** Measured concentrations are averages of two analyses.

TABLE 6A: INTERDAY PRECISION OF WR 254421 HUMAN URINE ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6 Measured Concentrations** (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
90.8	88.0	98.9	101	97.7	89.5	96.4	95.3	5.28	5.54	4.90
227	206	238	217	208	215	212	216	11.5	5.34	-4.85
1360	1270	1290	1240	1230	1270	1340	1273	39.3	3.09	-6.37
2270	2230	2370	1990	2260	2180	2130	2193	128.5	5.86	-3.38

TABLE 6B: INTRADAY PRECISION OF WR 254421 HUMAN URINE ASSAY

SPIKED CONC. (ng/ml)	SAMPLE NUMBER 1	2	3	4	5	6 Measured Concentrations (ng/ml)	MEAN (ng/ml)	S.D. (ng/ml)	Percent C.V.	Percent Error
90.8	117	99.3	99.2	102	85.4	103	101	10.1	10.0	11.2
227	216	212	215	215	206	230	216	7.92	3.67	-4.99
1360	1310	1270	1290	1260	1380	1260	1295	45.9	3.55	-4.78
2270	2060	2000	2100	2040	2080	2260	2090	90.1	4.31	-7.93

** Measured concentrations are averages of two analyses.

TABLE 7A: ACCURACY OF WR 6026 (FREE BASE) HUMAN URINE ASSAY (BLIND STUDY RESULTS)

Sample Number	Spiked Level (ng/ml)	Measured Level# (ng/ml)	Statistics (ng/ml)
6	2.60	*	Mean =
7		*	SD =
16		*	Percent CV =
22		*	Percent Bias =
1	5.2	7.12	Mean = 6.96
12		7.87	SD = 0.732
17		6.74	Percent CV = 10.5
23		6.12	Percent Bias = 33.9
2	15.5	12.5	Mean = 13.9
11		15.3	SD = 1.32
13		14.6	Percent CV = 9.51
24		13.0	Percent Bias = -10.6
3	56.2	52.2	Mean = 53.9
8		52.9	SD = 1.63
14		54.6	Percent CV = 3.03
21		55.8	Percent Bias = -4.14
4	78.7	72.3	Mean = 75.6
9		76.3	SD = 2.32
18		77.7	Percent CV = 3.07
20		76.2	Percent Bias = -3.91
5	101.2	97.1	Mean = 97.0
10		95.7	SD = 0.991
15		98.1	Percent CV = 1.02
19		97.2	Percent Bias = -4.13

n = 3, unless a chromatogram is determined to be unacceptable.

TABLE 7B: ACCURACY OF WR 211,789 (FREE BASE) HUMAN URINE ASSAY (BLIND STUDY RESULTS)

Sample Number	Spiked Level (ng/ml)	Measured Level# (ng/ml)	Statistics (ng/ml)
6	2.60	*	Mean =
7		*	SD =
16		*	Percent CV =
22		*	Percent Bias =
1	5.20	6.68	Mean = 6.97
12		7.92	SD = 0.720
17		7.04	Percent CV = 10.3
23		6.22	Percent Bias = 33.9
2	15.7	12.7	Mean = 13.9
11		13.8	SD = 1.03
13		15.2	Percent CV = 7.42
24		13.7	Percent Bias = -11.8
3	57.0	52.8	Mean = 54.8
8		54.2	SD = 1.60
14		55.7	Percent CV = 2.93
21		56.4	Percent Bias = -3.90
4	79.8	76.9	Mean = 77.2
9		76.8	SD = 0.655
18		78.2	Percent CV = 0.848
20		77.0	Percent Bias = -3.23
5	102.6	97.1	Mean = 98.1
10		97.4	SD = 1.94
15		101	Percent CV = 1.98
19		96.9	Percent Bias = -4.39

n = 3, unless a chromatogram is determined to be unacceptable.

TABLE 7C: ACCURACY OF WR 254,421 (FREE BASE) HUMAN URINE ASSAY (BLIND STUDY RESULTS)

Sample Number	Spiked Level (ng/ml)	Measured Level# (ng/ml)	Statistics (ng/ml)
6	5.0	*	Mean =
7		*	SD =
16		*	Percent CV =
22		*	Percent Bias =
1	50.1	56.2	Mean = 54.9
12		61.9	SD = 5.34
17		51.6	Percent CV = 9.72
23		50.0	Percent Bias = 9.63
2	150.3	124	Mean = 134
11		135	SD = 7.80
13		135	Percent CV = 5.81
24		143	Percent Bias = -10.7
3	544.1	513	Mean = 522
8		521	SD = 7.85
14		520	Percent CV = 1.51
21		532	Percent Bias = -4.15
4	761.6	745	Mean = 742
9		753	SD = 9.00
18		735	Percent CV = 1.21
20		734	Percent Bias = -2.61
5	979.4	935	Mean = 951
10		948	SD = 13.6
15		953	Percent CV = 1.43
19		968	Percent Bias = -2.90

n = 3, unless a chromatogram is determined to be unacceptable.

TABLE 8: STABILITIES OF WR 6026, WR 211,789 AND WR 254,421 IN HUMAN URINE

WR 6026

Spiked Concentration (ng/ml):	10.4	25.9	155	259
TIME STORED	WR 6026 Free Base Concentration of Samples Stored at -70°C# (ng/ml)			
0 days	9.53	19.8	144	187
1 day	12.2	25.7	132	186
2 days	11.5	25.1	151	203
4 days	9.81	20.9	137	185
1 week	10.9	24.0	139	197
2 weeks	9.82	22.0	131	218
3 weeks	7.22	24.2	145	241
1 month	8.40	20.5	160	239
4 months	10.7	23.6	153	229

Measured concentrations are averages of two analyses.

TABLE 8: STABILITIES OF WR 6026, WR 211,789 AND WR 254,421 IN HUMAN URINE (Continued)

WR 211,789

Spiked Concentration (ng/ml):	10.2	25.5	153	255
TIME STORED	WR 211,789 Free Base Concentration of Samples Stored at -70°C# (ng/ml)			
0 days	11.0	20.0	147	201
1 day	12.5	25.5	130	194
2 days	12.6	23.7	146	195
4 days	9.78	20.2	135	187
1 week	9.29	23.5	137	191
2 weeks	9.47	22.8	126	215
3 weeks	7.38	22.9	141	236
1 month	9.77	19.7	156	240
4 months	9.25	23.3	144	221

Measured concentrations are averages of two analyses.

TABLE 8: STABILITIES OF WR 6026, WR 211,789 AND WR 254,421 IN HUMAN URINE (Continued)

WR 254,421

Spiked Concentration (ng/ml):	100	250	1500	2500
TIME STORED	WR 254,421 Free Base Concentration of Samples Stored at -70°C# (ng/ml)			
0 days	96.0	205	1500	2110
1 day	121	261	1470	2095
2 days	102	252	1560	2070
4 days	96.7	223	1440	1940
1 week	87.3	226	1390	1940
2 weeks	98.4	221	1310	2190
3 weeks	96.6	233	1480	2400
1 month	97.9	219	1570	2370
4 months	96.2	232	1450	2110

Measured concentrations are averages of two analyses.

TABLE 9: AUTOSAMPLER STABILITY OF WR 6026, WR 211,789 AND WR 254,421 IN HUMAN URINE

WR 6026

Spiked Concentration (ng/ml):	10.4	25.9	155	259
TIME STORED	WR 6026 Free Base Concentration of Samples Stored at Room Temperature* (ng/ml)			
0 hours	11.1	26.4	159	238
24 hours	12.3	25.7	146	193
48 hours	14.5	26.6	149	224

WR 211,789

Spiked Concentration (ng/ml):	10.2	25.5	153	255
TIME STORED	WR 211,789 Free Base Concentration of Samples Stored at Room Temperature * (ng/ml)			
0 hours	10.5	25.7	142	229
24 hours	11.5	24.4	138	183
48 hours	11.1	25.9	139	205

WR 254,421

Spiked Concentration (ng/ml):	100	250	1500	2500
TIME STORED	WR 254,421 Free Base Concentration of Samples Stored at Room Temperature* (ng/ml)			
0 hours	114	250	1480	2280
24 hours	112	253	1300	1730
48 hours	115	260	1300	1900

*Measured concentrations are averages of two analyses.

LABORATORY METHODOLOGY FOR PRIMAQUINE FREE BASE AND CARBOXYLATED METABOLITE HUMAN PLASMA ASSAY,* STUDY REPORT 23

A. INSTRUMENTS

1. Refrigerated Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Altex Model 100A Solvent Delivery Module (Beckman Instruments Inc., Berkeley, CA) or equivalent.
3. Shimadzu SPD-10AV UV Detector (Shimadzu Scientific Instruments, Columbus, MD) or equivalent.
4. Hewlett-Packard Reporting Integrator #3390A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. WR 002,975AW (primaquine diphosphate) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BJ 08241, expiration date not available.
4. WR 249,725 (primaquine carboxylated metabolite) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BM 17852, expiration date not available.
5. Mebendazole (gift from Dr. David Kirn).
6. Phosphoric acid (85%) (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Tetramethylammonium Chloride (TMACl) (Fisher Scientific, Fair Lawn, NJ).
9. Ethyl Acetate (Fisher Scientific, Fair Lawn, NJ).
10. Formic acid (Sigma Chemical Co., St. Louis, MO).

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* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

11. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

C. ASSAY CONDITIONS

1. DETECTOR

   Settings
   Wavelength: 280 nm
   Range: 0.0050

2. COLUMN

   Axxiom ODS, 5 µm particle size, 4.6 x 250 mm
   (Richard Scientific, Novato, CA).

3. SOLVENT SYSTEM

   $CH_3OH/CH_3CN/H_2O$ (33:18.7:48.3, v/v/v) with 0.20% TMACl and 0.13% H_3PO_4, pH = 5.05 (apparent pH adjusted with 85% H_3PO_4).

4. FLOW RATE

   1.0 ml/min

5. STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator (in amber bottles, protected against exposure to light) and are discarded 6 months after the preparation date).

a. Primaquine diphosphate.

			Preparation date: 3/17/94		
Solution Type	Weight of Standard (mg)	Purity Factor*	Solvent Volume (ml)	Solvent	Free Base Conc. (µg/ml)
Standard Curve	18.702	0.5696	18.702	50% MeOH	570
Precision	23.264	0.5696	23.264	50% MeOH	570

*= Molecular weights of primaquine free base/primaquine diphosphate

b. Carboxylated metabolite.

			Preparation date: 3/17/94		
Solution Type	Weight of Standard (mg)	Purity Factor	Solvent Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	22.952	1	22.952	methanol	1000
Precision	22.482	1	22.482	methanol	1000

c. Mebendazole (Internal Standard).

Preparation date: 3/17/94

Solution Type	Weight of Standard (mg)	Purity Factor	Solvent Volume (ml)	Solvent	Conc. (µg/ml)
Internal Std	10.769	1	10.769	8.12% formic acid	1000

6. WORKING SOLUTIONS - Store solution in a 4°C refrigerator, protect against exposure to light and discard within 6 months.

a. Primaquine diphosphate.

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Free Base Conc. (µg/ml)
Standard Curve	570	1:10	50% MeOH	57.0
Precision	570	1:10	50% MeOH	57.0

b. Carboxylated metabolite.

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
Standard Curve	1000	1:10	50% MeOH	100
Precision	1000	1:10	50% MeOH	100

c. Combined primaquine diphosphate (free base concentration)/ carboxylated metabolite solutions.

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Compound	Volumes Combined (ml)	Conc. (µg/ml)
Standard Curve	57.0	1:2	primaquine	10	28.5
	100	1:2	carboxy	10	50.0
Precision	57.0	1:2	primaquine	10	28.5
	100	1:2	carboxy	10	50.0

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Compound	Solvent	Conc. (µg/ml)
Standard Curve	28.5	1:10	primaquine	50%	2.85
	50.0		carboxy	MeOH	5.00
Precision	28.5	1:10	primaquine	50%	2.85
	50.0		carboxy	MeOH	5.00

d. Mebendazole (Internal Standard).

Solution Type	Conc. Diluted (µg/ml)	Dilution Ratio	Solvent	Conc. (µg/ml)
Internal Std.	1000	3:2000	water	1.50

7. RETENTION TIMES (subject to change depending on temperature and column performance).

   a. Primaquine as free base - 11.0 min
   b. Carboxylated metabolite - 22.5 min
   c. Mebendazole (Internal Standard) - 14.5 min

8. BLANK HUMAN PLASMA

   Human plasma (CPD or CPDA-1 as anticoagulant) is obtained from the San Francisco Irwin Memorial Blood Bank.

9. INJECTION VOLUME: Samples that are expected to have high primaquine free base or carboxylated metabolite concentrations (i.e. high standard curve calibrators, high concentration control samples, and sponsor samples shown or expected to be near C_{peak}) are injected at the low end of the volume range.

   10-25 µl

10. QUANTITATION

    By peak height ratio of drug peak and metabolite peak relative to internal standard peak. Standard curves calculated by weighted linear regression where weights = 1/y.

11. LOWER LIMIT OF QUANTITATION OF METHOD (LLOQs were determined as the primaquine (free base) or carboxylated metabolite standard curve concentrations at which the signal to noise ratios were at least 3 to 1 and was based on the interday and intraday low point validation and on standard curve calibrator results.)

    28.5 ng/ml primaquine (free base) in plasma.

    20.0 ng/ml carboxylated metabolite in plasma.

12. SAMPLE VOLUME MEASUREMENT

    Plasma sample volumes were measured with a variable volume Eppendorf pipetter.

13. WISP OPERATING TEMPERATURE

    Refrigerated WISP temperature was less than or equal to 10°C.

14. SAMPLE EVAPORATION: Extracted samples are evaporated in a N-EVAP® Model 112 (Organomatic Assoc, Inc., S. Berlin, MA) by passing N_2 over the sample. The samples do not sit in water during evaporation.

D. SAMPLE STORAGE

All samples are to be kept frozen at -70°C before analysis and thawed at room temperature for preparation (within 30 min) and analysis, unless otherwise specified.

E. SAMPLE PREPARATION

1. If frozen, thaw human plasma sample at room temperature and vortex for 1 min. Pipet 0.500 ml of human plasma sample into a screw cap glass culture tube.
2. Spike standard curve samples with 00,* 0,** 0.2, 0.5, 1, 2, 3, 4, 5, 8, 15, 25, or 50 µl of 28.5 µg/ml primaquine diphosphate (free base concentration) and 50.0 µg/ml carboxylated metabolite working solution to make standard curves. Since 0.500 ml plasma samples are assayed, this procedure is equivalent to making standard curve samples with primaquine free base concentrations corresponding to 00, 0, 28.5, 57.0, 114, 171, 228, 285, 456, 854, 1420, and 2850 ng/ml and carboxylated metabolite concentrations corresponding to 00, 0, 20.0, 50.0, 100, 200, 300, 400, 500, 800, 1500, 2500, and 5000 ng/ml. Vortex 10 s.
3. Add 100 µl of internal standard (mebendazole = 1.50 µg/ml) solution. Vortex 10 s.
4. Add 3.0 ml of ethyl acetate.
5. Cap tube, and vortex 30 s, twice at speed 5 (VWR Multitube Vortexer). Centrifuge at 3000 *g* for 10 min.
6. Freeze aqueous layer in a dry ice/methanol bath and transfer organic phase into a 12x75 mm culture tube. Evaporate organic layer to dryness under nitrogen.
7. Add 2.0 ml of ethyl acetate to the aqueous layer. Repeat steps 5 and 6.
8. Reconstitute with 100 µl of 40% acetonitrile solution, vortex 1 min, and centrifuge at 3000 *g* for 10-15 min.
9. Transfer to WISP vial and inject 10-25 µl onto HPLC column.

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* 00 = Sample with no drug and no internal standard.
** 0 = Sample with no drug but with internal standard.

F. QUALITY CONTROL

1. Content and frequency of blanks

   No special blank was used except for the standard curve blank.

2. Pipette Calibration

   See ASOP 2C-1.1.

3. Balance Calibration

   See ASOP 2C-2.1.

G. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at four different concentrations by comparing the primaquine (as free base) and carboxylated metabolite to internal standard peak height ratios in reference samples to the peak height ratios in plasma. Plasma (0.5 ml) samples were spiked with primaquine (as free base) and carboxylated metabolite, then prepared as described above in "Sample Preparation" steps 3-9, except that in step 6; the sample was not evaporated to dryness and after step 7; 4 ml of ethyl acetate was taken, the internal standard was added, the samples vortexed 30 s, and the sample was evaporated to dryness. Reference samples were generated by spiking 5 ml of ethyl acetate with drug and metabolite to correspond with plasma samples, removing 4 ml of the resulting solutions, adding internal standard, evaporating to dryness then following steps 8 and 9 of "Sample Preparation."

H. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 0.5 ml plasma specimens with primaquine (as free base) and carboxylated metabolite working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Precision Samples

	Volume Spiked (µl)	Primaquine Free Base Spiking Solution Concentration (µg/ml)	Carboxylated Metabolite Spiking Solution Concentration (µg/ml)	Plasma Volume (ml)	Nominal Primaquine Free Base Concentration (ng/ml)	Nominal Carboxylated Metabolite Concentration (ng/ml)
X-Lo	1.5	28.5	50.0	0.5	85.4	150
Low	3	28.5	50.0	0.5	171	300
Med.	8	28.5	50.0	0.5	456	800
Hi	25	28.5	50.0	0.5	1420	2500

I. GENERATION OF STABILITY SAMPLES

1. Long term stability samples were generated by spiking pooled human plasma samples as shown below.

	Volume Spiked (µl)	Primaquine Free Base Spiking Solution Concentration (µg/ml)	Carboxylated Metabolite Spiking Solution Concentration (µg/ml)	Plasma Volume (ml)	Nominal Primaquine Free Base Concentration (ng/ml)	Nominal Carboxylated Metabolite Concentration (ng/ml)
	For -20°C Samples					
X-Lo	45	28.5	50.0	14.955	85.4	150
Low	90	28.5	50.0	14.910	171	300
Med.	240	28.5	50.0	14.760	456	800
Hi	750	28.5	50.0	14.250	1420	2500
	For -70°C Samples					
X-Lo	30	28.5	50.0	9.970	85.4	150
Low	60	28.5	50.0	9.940	171	300
Med.	160	28.5	50.0	9.840	456	800
Hi	500	28.5	50.0	9.500	1420	2500

2. Benchtop stability samples were generated by spiking pooled human plasma samples as shown below.

	Volume Spiked (µl)	Primaquine Free Base Spiking Solution Concentration (µg/ml)	Carboxylated Metabolite Spiking Solution Concentration (µg/ml)	Plasma Volume (ml)	Nominal Primaquine Free Base Concentration (ng/ml)	Nominal Carboxylated Metabolite Concentration (ng/ml)
X-Lo	15	28.5	50.0	4.985	85.4	150
Low	30	28.5	50.0	4.97	171	300
Med.	80	28.5	50.0	4.92	456	800
Hi	250	28.5	50.0	4.75	1420	2500

3. System stability samples were generated by spiking 0.5 ml human plasma specimens with primaquine (as free base) and carboxylated metabolite working solution as shown in Section H, "Generation of Precision Samples."

4. The effect of repeated freeze and thaw cycles on stability of primaquine (as free base) and carboxylated metabolite in human plasma samples was determined as described in laboratory SOP 2D-3.1, Section G.2. Freeze/thaw samples at Hi and Low concentrations were generated as described above for precision samples and in laboratory SOP 2D-3.1, Section G.2.

J. RESULTS

1. STANDARD CURVE

Chromatograms for each point in representative standard curves for primaquine (as free base) and carboxylated metabolite appear in Figure 3. Peak height ratios and corresponding standard curve concentrations for these calibrators appear in Table 1.

2. STUDY STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appears in Table 2

3. LOW POINT VALIDATION

The 6 back calculated lowest standard calibrator concentrations that were obtained in the interday precision-accuracy study were used as the interday minimum quantitation limit data. Results obtained for 6 lowest standard calibrator samples run with a standard curve were used as the intraday minimum quantitation limit data. Results appear in Table 3.

4. INTRA- AND INTERDAY PRECISION

Results for precision samples generated as described in Section H above were used for evaluations that appear in Tables 2 and 3.

5. RECOVERY

Results for recovery samples generated as described in Section G above were used for the evaluation that appears in Table 5.

6. STABILITY

a. Long term stability results appear in Tables 7 and 8.

b. Bench top stability results appear in Table 9.

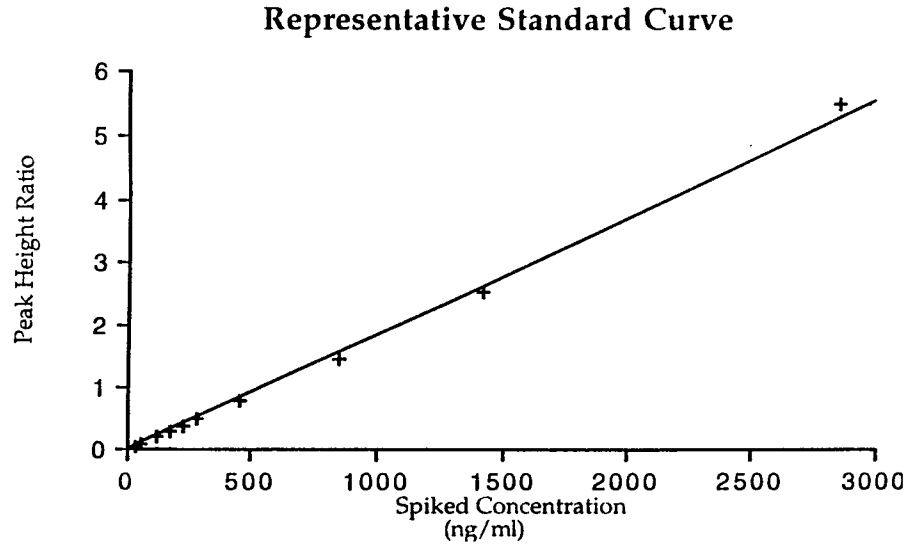
c. System (prepared sample) stability results appear in Table 10.

d. Freeze/thaw stability results appear in Table 11.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR PRIMAQUINE FREE BASE IN HUMAN PLASMA ASSAY, STUDY REPORT 23

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
	0	0	*
14.2	28.5	0.052	33.0
28.5	57.0	0.101	59.5
57.0	114	0.202	114
85.4	171	0.292	163
114	228	0.392	217
142	285	0.503	277
228	456	0.806	441
427	854	1.483	807
712	1420	2.570	1390
1420	2850	5.486	2970

Regression equations:***
$y = 0.001850x - 0.009103$, $r^2 = 0.9979$

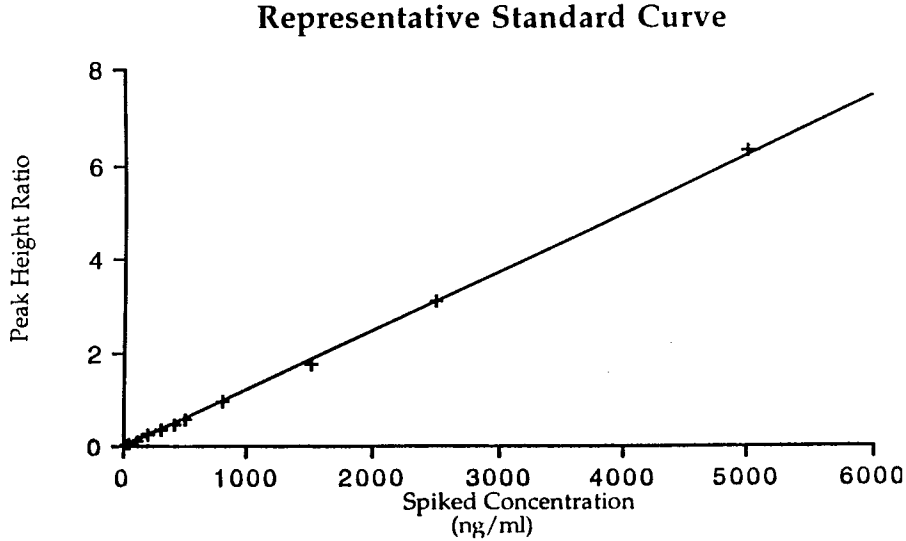


* Into 0.5 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression, where weight = 1/y.

TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR CARBOXY-PRIMAQUINE METABOLITE IN HUMAN PLASMA ASSAY, STUDY REPORT 23

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
	0	0	*
10.0	20.0	0.035	23.2
25.0	50.0	0.067	48.9
50.0	100	0.127	97.2
100	200	0.264	207
150	300	0.373	295
200	400	0.480	381
250	500	0.620	494
400	800	0.996	796
750	1500	1.809	1450
1250	2500	3.121	2510
2500	5000	6.316	5080

Regression equations:***
$y = 0.001243x + 0.006200$, $r^2 = 0.9995$



* Into 0.5 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression, where weight = 1/y.

TABLE 2: PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS FOR PRIMAQUINE HUMAN PLASMA ASSAY

Spiked Concentration (ng/ml)	n	Mean (ng/ml)	Standard Deviation (ng/ml)	Percent C.V.	Percent R.E.
		Primaquine Free Base			
28.5	7	33.2	2.11	6.34	16.6
57	7	58.1	2.74	4.73	1.85
114	7	112	4.38	3.90	-1.63
171	7	164	3.26	1.99	-4.34
228	7	219	8.08	3.68	-3.76
285	7	271	8.50	3.13	-4.76
456	7	440	13.4	3.04	-3.48
854	7	829	20.4	2.46	-2.96
1420	7	1420	32.0	2.26	-0.20
2850	7	2930	71.3	2.43	2.76
		Carboxylated Metabolite			
20	7	19.7	2.92	14.8	-1.50
50	7	49.8	3.45	6.93	-0.37
100	7	101	3.96	3.92	1.01
200	7	205	7.26	3.54	2.50
300	7	300	8.59	2.87	-0.048
400	7	405	15.2	3.76	1.14
500	7	505	14.5	2.87	1.00
800	7	809	21.8	2.69	1.07
1500	7	1460	66.2	4.54	-2.76
2500	7	2470	25.0	1.01	-1.09
5000	7	5050	56.8	1.12	1.06

TABLE 3: LOWER LIMITS OF QUANTITATION OF THE PRIMAQUINE HUMAN PLASMA ASSAY

Primaquine Free Base

Spiked Concentration	(28.5 ng/ml)	(28.5 ng/ml)
Sample	Measured Concentrations (ng/ml)	
	Interday	Intraday
1	33.6	34.9
2	31.3	35.5
3	35.1	30.3
4	35.6	32.6
5	33.0	34.3
6	34.3	35.5
Mean	33.8	33.9
Standard Deviation	1.56	2.05
Percent CV	4.60	6.04
Percent R.E.	18.7	18.8

Carboxylated Metabolite

Spiked Concentration	(20.0 ng/ml)	(20.0 ng/ml)
Sample	Measured Concentrations (ng/ml)	
	Interday	Intraday
1	20.4	20.0
2	18.9	18.5
3	16.0	bc
4	16.1	18.5
5	23.2	17.0
6	23.0	16.3
Mean	19.6	18.1
Standard Deviation	3.19	1.45
Percent CV	16.3	8.01
Percent R.E.	-2.00	-9.70

TABLE 4: PRECISION OF PRIMAQUINE HUMAN PLASMA ASSAY

Interday Precision Primaquine

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 85.4	171	456	1420
		Measured Concentrations (ng/mL)			
1	1	85.8	157	442	1320
	2	85.8	157	446	1420
2	1	81	166	443	1400
	2	85.3	173	436	1380
3	1	82.2	158	426	1390
	2	82.2	164	426	1390
4	1	91.5	167	441	1450
	2	81.5	161	426	1340
5	1	87.1	163	448	1410
	2	82.2	162	444	1390
6	1	83.4	161	459	1440
	2	82.8	160	431	1400
n		12	12	12	12
Mean		84.2	162	439	1390
S.D.		3.02	4.64	10.3	36.8
Percent C.V.		3.58	2.86	2.34	2.64
Percent R.E.		-1.37	-5.02	-3.73	-1.82

Intraday Precision Primaquine

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 85.4	171	456	1420
		Measured Concentrations (ng/mL)			
7	1	84.4	166	453	1440
	2	83.1	159	437	1460
	3	81.9	164	446	1410
	4	83.8	165	453	1400
	5	84.4	160	458	1440
	6	85.0	176	434	1460
n		6	6	6	6
Mean		83.8	165	447	1435
S.D.		1.12	6.07	9.62	25.1
Percent C.V.		1.34	3.68	2.15	1.75
Percent R.E.		-1.91	-3.51	-2.01	1.06

TABLE 5: PRECISION OF PRIMAQUINE HUMAN PLASMA ASSAY

Interday Precision Carboxy-Primaquine

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 150	300	800	2500
		Measured Concentrations (ng/mL)			
1	1	149	292	804	2490
	2	160	306	806	2540
2	1	161	298	816	2540
	2	157	316	791	2550
3	1	153	307	847	2660
	2	151	314	867	2570
4	1	158	298	824	2460
	2	157	289	816	2340
5	1	146	288	818	2480
	2	153	298	793	2450
6	1	139	296	810	2500
	2	141	288	763	2440
n		12	12	12	12
Mean		152	299	813	2500
S.D.		7.19	9.68	26.6	79.8
Percent C.V.		4.73	3.23	3.27	3.19
Percent R.E.		1.39	-0.278	1.61	0.0667

Intraday Precision Carboxy-Primaquine

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 150	300	800	2500
		Measured Concentrations (ng/mL)			
7	1	152	294	789	2220
	2	153	301	733	2440
	3	147	309	794	2320
	4	152	328	806	2330
	5	136	324	834	2470
	6	159	320	760	2610
n		6	6	6	6
Mean		149	324	800	2470
S.D.		11.8	4.00	37.4	140
Percent C.V.		7.91	1.23	4.67	5.67
Percent R.E.		-0.67	8.00	0.00	-1.20

TABLE 6: RECOVERIES OF PRIMAQUINE AND CARBOXYLATED METABOLITE FROM HUMAN PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	(ng/ml)	PEAK HEIGHT RATIO REFERENCE	PLASMA	AVERAGE PERCENT RECOVERY
Primaquine					
1	High	1420	5.800	5.821	94.6
2			5.969	5.639	
3			6.423	5.750	
Mean (± SD)			6.064 ±0.322	5.737 ±0.092	
1	Medium	456	1.798	1.776	97.7
2			1.782	1.780	
3			1.862	1.763	
Mean (± SD)			1.814 ±0.042	1.773 ±0.009	
1	Low	171	0.629	0.659	104.0
2			0.628	0.667	
3			0.639	0.645	
Mean (± SD)			0.632 ±0.006	0.657 ±0.011	
1	X Low	85.4	0.293	0.306	107.4
2			0.305	0.317	
3			0.277	0.317	
Mean (± SD)			0.292 ±0.014	0.313 ±0.006	
OVERALL AVERAGE RECOVERY =					100.9
Carboxylated metabolite					
1	High	2500	8.878	6.681	72.9
2			8.915	6.645	
3			9.604	6.653	
Mean (± SD)			9.132 ±0.409	6.660 ±0.019	
1	Medium	800	2.851	2.128	73.6
2			2.849	2.089	
3			2.851	2.078	
Mean (± SD)			2.850 ±0.001	2.098 ±0.026	
1	Low	300	1.073	0.840	76.8
2			1.071	0.840	
3			1.069	0.786	
Mean (± SD)			1.071 ±0.002	0.822 ±0.031	
1	X Low	150	0.532	0.393	76.9
2			0.562	0.417	
3			0.527	0.436	
Mean (± SD)			0.540 ±0.019	0.415 ±0.022	
OVERALL AVERAGE RECOVERY =					75.0

TABLE 7: LONG TERM FREEZER STORAGE STABILITIES OF THE PRIMAQUINE HUMAN PLASMA ASSAY

Primaquine Concentration in Human Plasma Stored at -20°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	85.4	171	456	1420
TIME STORED				
0 days	84.1	168	451	1450
1 day	77.7	172	420	1380
2 days	85.2	153	424	1360
8 days	86.8	163	438	1340
2 weeks	79.9	162	424	1330
3 weeks	74.8	164	450	1440
1 month	86.2	163	434	1390
2 months	83.5	140	419	1180
3 months	88.6	154	412	1420

Carboxy Metabolite Concentration in Human Plasma Stored at -20°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	150	300	800	2500
TIME STORED				
0 days	143	293	766	2450
1 day	144	290	742	2370
2 days	135	255	741	2060
8 days	149	298	769	2460
2 weeks	142	286	749	2280
3 weeks	140	294	778	2060
1 month	138	294	730	2400
2 months	152	245	739	2120
3 months	141	272	780	2520

*Measured concentrations are averages of two analyses.

TABLE 8: LONG TERM FREEZER STORAGE STABILITIES OF THE PRIMAQUINE HUMAN PLASMA ASSAY

Primaquine Concentration in Human Plasma Stored at -70°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	85.4	171	456	1420
TIME STORED				
0 days	85.1	166	430	1400
8 days	83.7	162	424	1410
13 days	87.9	168	436	1420
19 days	83.4	164	419	1460
2 months	91.2	176	496	1450
3 months	73.3	150	406	1370
6 months	78.0	153	388	1430

Carboxy Metabolite Concentration in Human Plasma Stored at -70°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	150	300	800	2500
TIME STORED				
0 days	156	308	800	2610
8 days	157	321	823	2440
13 days	153	303	811	2480
19 days	136	293	780	2590
2 months	153	309	778	2650
3 months	148	312	805	2540
6 months	161	326	813	2940

*Measured concentrations are averages of two analyses.

TABLE 9: BENCHTOP STABILITIES OF THE PRIMAQUINE HUMAN PLASMA ASSAY

Primaquine Concentration in Human Plasma Stored at Room Temperature

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	85.4	171	456	1420
TIME STORED				
0 hour	98.6	173	433	1340
2 hours	95.8	176	436	1330
4 hours	98.0	201	480	1480
6 hours	92.5	173	459	1490

Carboxy Metabolite Concentration in Human Plasma Stored at Room Temperature

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	150	300	800	2500
TIME STORED				
0 hour	147	292	752	2210
2 hours	151	302	713	2200
4 hours	152	302	725	2320
6 hours	158	304	762	2300

TABLE 10: SYSTEM STABILITIES OF THE PRIMAQUINE HUMAN PLASMA ASSAY

Primaquine Concentration of Prepared Samples Stored at Less Than or Equal to 10°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	85.4	171	456	1420
TIME STORED				
0 hour	85.3	167	481	1450
48 hours	91.2	176	496	1450

Carboxy Metabolite Concentration of Prepared Samples Stored at Less Than or Equal to 10°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	150	300	800	2500
TIME STORED				
0 hour	159	299	880	2520
48 hours	160	320	917	2550

TABLE 11: EFFECT OF REPEATED FREEZE AND THAW CYCLES IN THE PRIMAQUINE HUMAN PLASMA ASSAY

	Primaquine free base		Carboxylated metabolite	
Spiked Concentration	Low Concentration* (171 ng/ml)	High Concentration (1420 ng/ml)	Low Concentration* (300 ng/ml)	High Concentration (2500 ng/ml)
Cycle				
1	180	1330	312	2200
2	183	1140	304	2000
3	177	1250	295	2020
4	185	1570	278	2720
5	173	1350	272	2180

*Measured concentrations are averages of two analyses.

LABORATORY METHODOLOGY FOR GENTAMICIN/PAROMOMYCIN HUMAN PLASMA ASSAY, STUDY REPORT 24

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 717 (Waters Associates, Milford, MA) or equivalent.

2. LC-600 Shimadzu Pump (Shimadzu Corp., Kyoto, Japan) or equivalent. Two are required, one for mobile phase and one for post-column reagent.

3. Shimadzu RF 535 Fluorescence Detector (Shimadzu Corp., Kyoto, Japan) or equivalent.

4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

6. T-mixer, Valco (Altech, Berkeley, CA)

B. REAGENTS

1. Gentamicin sulfate (WR 073633), bottle no. BM 18591 (WRAIR, Washington D.C.).

2. Paromomycin sulfate (WR 035928), bottle no. BM 17861 (WRAIR, Washington D.C.).

3. Sisomicin (Internal Standard) (Sigma Chemical Co., St. Louis, MO).

4. Boric acid (Sigma Chemical Co., St. Louis, MO).

5. *o*-Pthaldialdehyde (Sigma Chemical Co., St. Louis, MO).

6. 2-Mercaptoethanol (Sigma Chemical Co., St. Louis, MO).

7. Sodium sulfate (Sigma Chemical Co., St. Louis, MO).

8. Sodium octane sulfonate (Sigma Chemical Co., St. Louis, MO).

9. Potassium hydroxide (Fisher Scientific, Fair Lawn, NJ).

10. Glacial acetic acid (Fisher Scientific, Fair Lawn, NJ).

*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

11. Perchloric acid (Fisher Scientific, Fair Lawn, NJ).

12. Methanol (Fisher Scientific, Fair Lawn, NJ).

C. ASSAY CONDITIONS

1. DETECTOR

   Settings

   Wavelengths: Excitation 340 nm; Emission-430 nm
   Range: 16

   Lamp: USHIO XENON, TYPE UXL-155-LCA(S-LC)

2. COLUMN

   Capcell C18 type SG 120Å, 5 µm particle size, 4.6 x 150 mm (Shiseido) or equivalent.

3. MOBILE PHASE SOLVENT SYSTEM

   16% CH_3CN, 0.2 M Na_2SO_4, 0.02 M sodium octanesulfonate, 0.1% acetic acid

4. POST COLUMN REAGENT (Dissolve *o*-phthalaldehyde in methanol. Add boric acid and potassium hydroxide to half the water, mix, add *o*-phthalaldehyde solution, mix, filter then add remaining water and mercaptoethanol. Store in 4°C refrigerator, use within 4 days and keep in an ice bucket with an ice pack when running the HPLC system.)

   Water/methanol/mercaptoethanol (99.4/0.5/0.1) (v/v/v), 0.201 molal boric acid, 0.218 molal KOH and 0.0008% *o*-phthalaldehyde.

5. FLOW RATE

   mobile phase: 1.0 ml/min

   post column reagent: 0.3 ml/min

6. STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and checked for deterioration by comparison to a newly made solution (solutions are discarded when a more than 10% change in the absolute peak height is observed or by 6 months after the preparation date).

a. WR 073633 (gentamicin sulfate) for precision expressed as the free base concentration.

Prep date: 2/21/95

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (mg/ml)
Standard Curve	15.56	0.607	10.13	mobile phase	1.00
Precision	15.56	0.607	10.11	mobile phase	1.00

*obtained from WRAIR

b. WR 035928 (paromomycin sulfate) for precision expressed as the free base concentration.

Prep date: 2/21/95

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (mg/ml)
Standard Curve	16.69	0.646	10.05	mobile phase	1.00
Precision	16.67	0.646	10.05	mobile phase	1.00

*obtained from WRAIR

c. Sisomicin (internal standard).

Prep date: 2/21/95

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (mg/ml)
Internal std.	11.50	1	11.5	mobile phase	1.00

7. WORKING SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and discarded when stock solutions were discarded or by 6 months after the preparation date).

a. High concentration combined gentamicin/paromomycin (as free bases) solutions.

Solution Type	Conc. Diluted (mg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	1.00	2.00 each	20	mobile phase	100
Precision	1.00	2.00 each	20	mobile phase	100

b. Low concentration combined gentamicin/paromomycin (as free bases) solutions.

Solution Type	Conc. Diluted (mg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	100	2.00	20	mobile phase	10.0
Precision	100	2.00	20	mobile phase	10.0

c. Sisomicin - Internal standard.

Solution Type	Conc. Diluted (mg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (mg/ml)
Internal std.	1.00	2.00	90	mobile phase	0.22

8. RETENTION TIMES (subject to change depending on temperature and column performance).

   a. Paromomycin - 7.0 min
   b. Gentamicin - 15.5 min
   c. Sisomicin (Internal Standard) - 10.0 min

9. BLANK PLASMA

   Human plasma (CPD or CPDA-1 as anticoagulant) was obtained from the San Francisco Irwin Memorial Blood Bank.

10. INJECTION VOLUME

    10 µl

11. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves are calculated by weighted linear regression where weights = 1/y.

12. LOWER LIMIT OF QUANTITATION OF METHOD (The lower limits of quantitation (LLOQ) of the assay of human plasma for gentamicin and paromomycin (as free bases) were based on the interday and intraday low point validation results, on standard curve calibrator results, and a minimum 2 to 1 signal to noise ratio.)

    0.100 µg/ml gentamicin (as free base) in human plasma
    0.100 µg/ml paromomycin (as free base) in human plasma

13. VOLUME MEASUREMENT

    Plasma sample volumes were measured with a 200 µl or a 1000 µl Gilson Pipetman. Hamilton syringes were used to measure standard and control solution volumes.

14. WISP OPERATING TEMPERATURE

    Room temperature.

15. COLUMN HEATER

Operating temperature: 40°C

16. REACTION COIL

Teflon knitted tube: 5 m, internal diameter; 0.3 mm

D. SAMPLE STORAGE

All samples were kept frozen at -70°C before analysis and thawed for preparation and analysis, unless specified otherwise.

E. SAMPLE PREPARATION

1. If frozen, vortex specimens for 20 seconds after sample thaws.
2. Pipet 0.2 ml of a plasma sample into a clean glass culture tube.
3. Spike standard curve samples as shown in Section G "Generation of Standard Curve Calibrators."
4. Add 20 µl of internal standard (0.022 mg/ml Sisomicin) solution.
5. Add 20 µl of perchloric acid. Vortex for 1 min.
6. Centrifuge 10 minutes at 3000 *g*.
7. Transfer supernatant to WISP vial and inject onto HPLC column.

F. QUALITY CONTROL

1. Content and frequency of blanks

   A blank plasma sample was prepared as described in "Sample Preparation" and assayed at least once for each standard curve in precision assays.

2. PIPETTE CALIBRATION

   See SOP 2C-1.2.

3. BALANCE CALIBRATION

   See SOP 2C-2.1

G. GENERATION OF STANDARD CURVE CALIBRATORS

A representative example of the generation of standard curve calibrators is shown in the table below. Spike blank plasma standard curve samples with mixed gentamicin/paromomycin (as free bases) solution to make a standard curve. This procedure is equivalent to addition of the masses of gentamicin and paromomycin (as free bases) shown below. Since 0.200 ml plasma samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 10s.

Generation of Gentamicin/Paromomycin (as free base)
Standard Curve Calibrators

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (µg/ml)
00*	0	-	0	0
0**	0	-	0	0
1	2	10	20	0.100
2	4	10	40	0.200
3	8	10	80	0.400
4	16	10	160	0.800
5	3	100	300	1.50
6	6	100	600	3.00
7	12	100	1200	6.00
8	24	100	2400	12.0

H. GENERATION OF PRECISION SAMPLES

Precision samples were generated by spiking 0.2 ml plasma specimens with control working solutions to make the gentamicin/ paromomycin (as free bases) concentrations shown.

Generation of Gentamicin/Paromomycin (as free bases)
Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (µg/ml)
X-Lo	4	10.0	0.2	0.200
Low	16	10.0	0.2	0.800
Med.	5	100	0.2	2.50
Hi	10	100	0.2	5.00

*00 = Sample with no drug and no internal standard.
*0 = Sample with no drug but with internal standard.

I. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at four different concentrations by comparing the gentamicin/paromomycin to internal standard peak height ratios in reference samples to the peak height ratios in plasma. Plasma (0.2 ml) samples were spiked with gentamicin/paromomycin (and vortexed) then prepared as described above in "Sample Preparation," except no standard curve is used. Reference samples were generated by spiking 0.2 ml mobile phase with gentamicin/paromomycin then preparing the sample as described above in "Sample Preparation," except no standard curve is used.

J. GENERATION OF STABILITY SAMPLES

1. Long term stability samples were generated by spiking pooled human plasma samples as shown below.

	Volume Spiked (µl)	Gentamicin/Paromomycin Free Base Spiking Solution Concentration (µg/ml)	Plasma Volume (ml)	Gentamicin Free Base Concentration (µg/ml)	Paromomycin Free Base Concentration (µg/ml)
X-Lo	60	100	14.940	0.400	0.400
Low	150	100	14.850	1.00	1.00
Med.	375	100	14.625	2.50	2.50
Hi	75	1000	14.925	5.00	5.00

2. Benchtop stability samples were generated by spiking pooled human plasma samples as shown below.

	Volume Spiked (µl)	Gentamicin Free Base Spiking Solution Concentration (µg/ml)	Plasma Volume (ml)	Gentamicin Free Base Concentration (µg/ml)	Paromomycin Free Base Concentration (µg/ml)
X-Lo	60	100	2.940	0.200	0.200
Low	24	100	2.976	0.800	0.800
Med.	75	100	2.925	2.50	2.50
Hi	150	100	2.850	5.00	5.00

3. System stability samples were generated by spiking 0.2 ml human plasma specimens with gentamicin/paromomycin (as free bases) working solution as shown in Section H, "Generation of Precision Samples."

4. The effect of repeated freeze and thaw cycles on stability of gentamicin/paromomycin in human plasma samples was determined as described in laboratory SOP 2D-3.1, Section G.2. Freeze/thaw samples at Hi and Low concentrations were generated as described above for benchtop stability samples and in laboratory SOP 2D-3.1, Section G.2.

K. VALIDATION RESULTS

1. STANDARD CURVE

   Chromatograms for each point in a representative standard curve for gentamicin/paromomycin appear in Figure 3. Peak height ratios for these calibrators appear in Table 1. Statistical parameters of plasma interday precision standard curve calibrators appear in Table 2.

2. INTRA- AND INTERDAY PRECISION

   Results for these evaluations appear in Table 3.

3. LLOQ

   Results for this evaluation appear in Table 4.

4. RECOVERY

   Results for this evaluation appear in Table 5.

5. STABILITY

   a. System Stability: Results appear in Table 6.

   b. Long Term Stability: Results appear in Table 7.

   c. Bench Top Stability: Results appear in Table 8.

   d. Freeze/Thaw Stability: Results appear in Table 9.

6. BLIND SAMPLE ANALYSIS

   Results appear in Table 10.

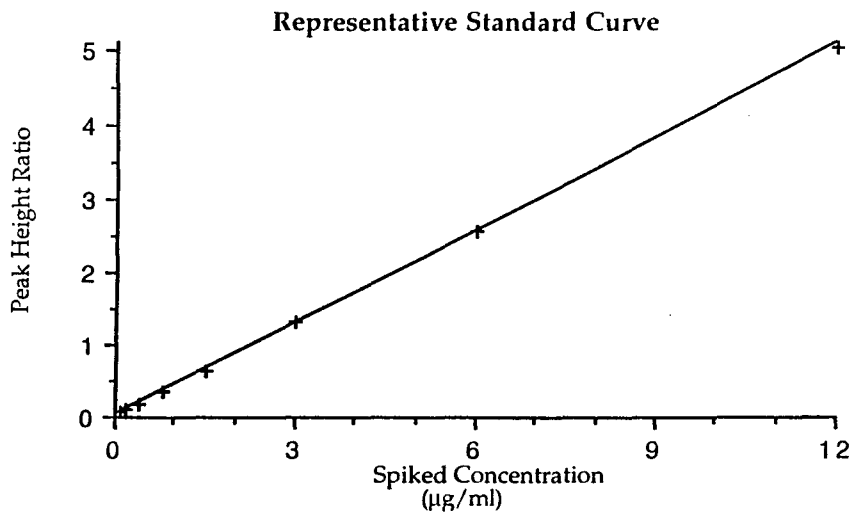
7. RAT PLASMA SHORT VALIDATION

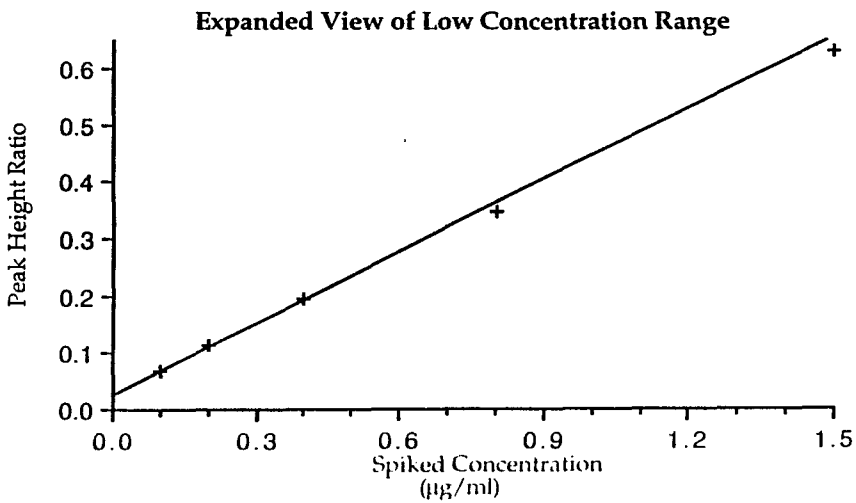
   Results appear in Tables 11-14.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR GENTAMICIN HUMAN PLASMA ASSAY, STUDY REPORT 24

SPIKED OR DILUTION AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (µg/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (µg/ml)
0	0	-	-
20	0.100	0.066	0.0987
40	0.200	0.113	0.211
80	0.400	0.195	0.406
160	0.800	0.347	0.768
300	1.50	0.634	1.45
600	3.00	1.309	3.06
1200	6.00	2.569	6.06
2400	12.0	5.037	11.9

Regression equation:***
$y = 0.420x + 0.0246$, $r^2 = 0.9997$



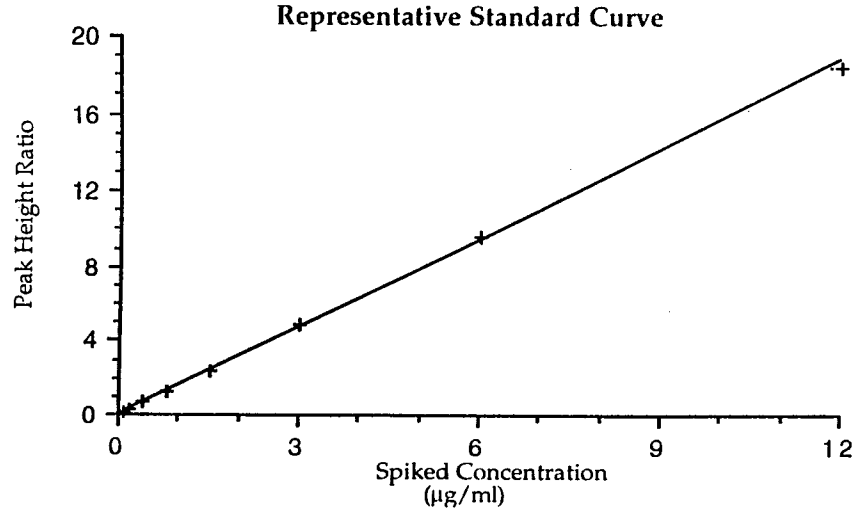


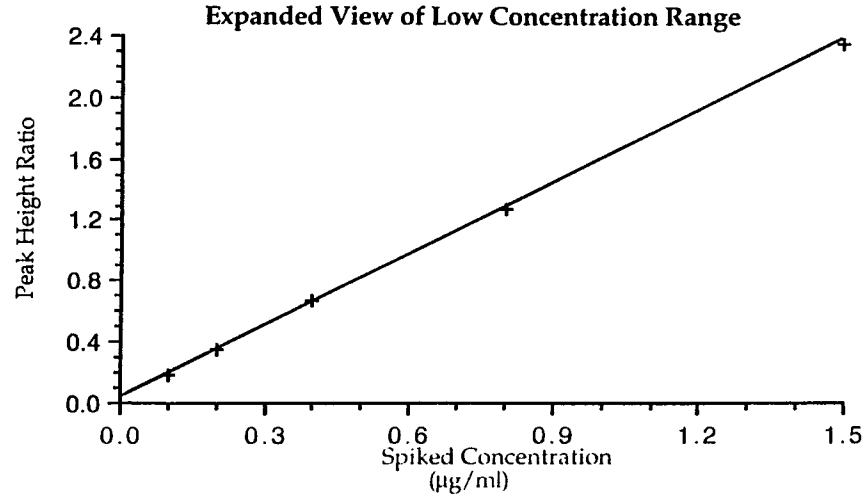
* In 0.2 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR PAROMOMYCIN HUMAN PLASMA ASSAY, STUDY REPORT 24

SPIKED OR DILUTION AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (µg/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (µg/ml)
0	0	-	-
20	0.100	0.181	0.957
40	0.200	0.346	0.201
80	0.400	0.665	0.405
160	0.800	1.275	0.794
300	1.50	2.347	1.48
600	3.00	4.951	3.14
1200	6.00	9.597	6.10
2400	12.0	18.515	11.8

Regression equation:***
$y = 1.57x + 0.0310$, $r^2 = 0.9994$





* In 0.2 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 2A: PRECISION STANDARD CURVE DATA FOR GENTAMICIN HUMAN PLASMA ASSAY, STUDY REPORT 24

Gentamicin Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
2/23/95	interge1	0.46760957	-0.0048544	0.99874855
2/28/95	intrage	0.4503625	0.00545335	0.99982051
3/2/95	day1gent	0.43440038	0.00910677	0.99948823
3/3/95	day2gent	0.43067307	0.00340248	0.9990281
3/4/95	day3gent	0.41962867	0.02458418	0.99973472
3/4/95	inter3ge	0.42586435	-0.0015976	0.99909062

Gentamicin Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml)							
	0.100	0.200	0.400	0.800	1.50	3.00	6.00	12.0
	Back Calculated Concentration (µg/ml)							
interge1	0.113	0.184	0.391	0.740	1.53	3.14	6.18	11.7
intrage	0.0967	0.197	0.399	0.852	1.49	3.01	5.96	12.0
day1gent	0.119	0.184	0.382	0.787	1.46	3.06	6.12	11.9
day2gent	0.106	0.206	0.410	0.772	1.47	2.85	5.89	12.3
day3gent	0.0987	0.211	0.406	0.768	1.45	3.06	6.06	11.9
inter3ge	0.107	0.203	0.408	0.772	1.43	2.84	6.17	12.1
n	6	6	6	6	6	6	6	6
Mean	0.107	0.198	0.399	0.782	1.47	2.99	6.06	12.0
SD	0.00843	0.0114	0.0110	0.0376	0.0349	0.122	0.117	0.204
Percent CV	7.88	5.76	2.76	4.81	2.37	4.08	1.93	1.70
Percent RE	+7.00	-1.00	-0.250	-2.25	-2.00	-0.333	+1.00	0

TABLE 2B: PRECISION STANDARD CURVE DATA FOR PAROMOMYCIN HUMAN PLASMA ASSAY, STUDY REPORT 24

Paromomycin Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
2/23/95	inter-1	1.98288996	0.04673078	0.99970962
2/28/95	intrapa	1.70096037	0.03214057	0.99932455
3/2/95	day1pa	1.46449057	-0.0329994	0.99893475
3/3/95	day2paro	1.36286881	-0.0583854	0.99897777
3/4/95	day3paro	1.56709206	0.03098221	0.99941396
3/4/95	inter3pa	1.34555578	-0.0674372	0.99870615

Paromomycin Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml)							
	0.100	0.200	0.400	0.800	1.50	3.00	6.00	12.0
	Back Calculated Concentration (µg/ml)							
inter-1	0.0990	0.214	0.401	0.755	1.47	3.08	5.99	12.0
intrapa	0.0887	0.196	0.401	0.867	1.55	3.10	5.99	11.8
day1pa	0.0881	bc	0.435	0.861	1.48	3.16	6.02	11.8
day2paro	0.0883	0.234	0.437	0.822	1.51	2.87	5.90	12.2
day3paro	0.0957	0.201	0.405	0.794	1.48	3.14	6.10	11.8
inter3pa	0.116	0.192	0.365	0.770	1.45	2.88	6.26	12.0
n	6	5	6	6	6	6	6	6
Mean	0.0960	0.207	0.407	0.812	1.49	3.04	6.04	11.9
SD	0.0108	0.0170	0.0265	0.0466	0.0352	0.130	0.124	0.163
Percent CV	11.3	8.21	6.51	5.74	2.36	4.28	2.05	1.37
Percent RE	-4.00	+3.50	+1.75	+1.50	-0.667	+1.33	+0.667	-0.833

bc = unacceptable chromatogram.

TABLE 3A: PRECISION OF GENTAMICIN HUMAN PLASMA ASSAY

Interday Precision Gentamicin

Validation Run	QC Sample No.	Spiked Concentrations (µg/mL) 0.200	0.800	2.50	5.00
		Measured Concentrations (µg/mL)			
interge1	1	0.188	0.808	2.50	5.07
	2	0.222	0.823	2.54	5.01
intrage	1	0.203	0.796	2.52	4.87
	2	0.199	0.796	2.40	5.11
day1gent	1	0.189	0.822	2.40	5.38
	2	0.198	0.833	2.37	5.12
day2gent	1	0.194	0.756	2.41	4.84
	2	0.192	0.749	2.37	4.80
day3gent	1	0.218	0.816	2.43	5.26
	2	0.156	0.802	2.56	5.12
inter3ge	1	0.194	0.715	2.25	4.53
	2	0.201	0.743	2.23	4.35
n		12	12	12	12
Mean		0.196	0.788	2.42	4.96
SD		0.0165	0.0379	0.105	0.295
Percent CV		8.42	4.81	4.34	5.95
Percent RE		-2.00	-1.50	-3.20	-0.800

Intraday Precision Gentamicin

Validation Run	QC Sample No.	Spiked Concentrations (µg/mL) 0.200	0.800	2.50	5.00
		Measured Concentrations (µg/mL)			
intrage	1	0.192	0.789	2.27	4.95
	2	0.183	0.809	2.44	4.95
	3	0.190	0.803	2.38	4.73
	4	0.190	0.801	2.33	4.70
	5	0.183	0.792	2.36	4.74
	6	0.177	0.758	2.44	5.11
n		6	6	6	6
Mean		0.186	0.792	2.37	4.86
SD		0.00578	0.0182	0.0657	0.165
Percent CV		3.11	2.30	2.77	3.40
Percent RE		-7.00	-1.00	-5.20	-2.80

TABLE 3B: PRECISION OF PAROMOMYCIN HUMAN PLASMA ASSAY

Interday Precision Paromomycin

Validation Run No.	QC Sample No.	Spiked Concentrations (µg/mL) 0.200	0.800	2.50	5.00
		Measured Concentrations (µg/mL)			
inter-1	1	0.184	0.807	2.67	5.17
	2	0.197	0.837	2.58	5.05
intrapa	1	0.217	0.895	2.78	5.40
	2	0.195	0.876	2.64	5.69
day1pa	1	0.175	0.862	2.23	5.21
	2	0.186	0.877	2.40	5.05
day2paro	1	0.183	0.769	2.36	4.70
	2	0.181	0.749	2.30	4.65
day3paro	1	0.192	0.846	2.53	5.41
	2	0.202	0.831	2.66	5.24
inter3pa	1	0.186	0.703	2.17	4.36
	2	0.193	0.734	2.14	4.18
n		12	12	12	12
Mean		0.191	0.816	2.46	5.01
SD		0.0112	0.0629	0.217	0.451
Percent CV		5.86	7.71	8.82	9.00
Percent RE		-4.50	+2.00	-1.60	+0.200

Intraday Precision Paromomycin

Validation Run No.	QC Sample No.	Spiked Concentrations (µg/mL) 0.200	0.800	2.50	5.00
		Measured Concentrations (µg/mL)			
intrapa	1	0.188	0.845	2.41	5.05
	2	0.186	0.837	2.45	5.13
	3	0.189	0.827	2.40	4.73
	4	0.187	0.810	2.33	4.73
	5	0.184	0.792	2.33	4.72
	6	0.173	0.781	2.21	5.05
n		6	6	6	6
Mean		0.185	0.815	2.36	4.90
SD		0.00589	0.0254	0.0853	0.194
Percent CV		3.18	3.12	3.61	3.96
Percent RE		-7.50	+1.87	-5.60	-2.00

TABLE 4: LOWER LIMIT OF QUANTITATION OF THE HUMAN PLASMA ASSAY FOR GENTAMICIN/PAROMOMYCIN

Gentamicin

Spiked Concentration	0.100 µg/ml	0.100 µg/ml
Sample	Measured Concentrations (µg/ml)	
	Interday	Intraday
1	0.113	0.111
2	0.0967	0.0799
3	0.119	0.0799
4	0.106	0.0732
5	0.0987	0.0799
6	0.107	0.0866
Mean	0.107	0.0851
SD	0.00843	0.0134
Percent CV	7.88	15.7
Percent RE	7.00	-14.9

Paromomycin

Spiked Concentration	0.100 µg/ml	0.100 µg/ml
Sample	Measured Concentrations (µg/ml)	
	Interday	Intraday
1	0.0990	0.101
2	0.0887	0.0851
3	0.0881	0.0877
4	0.0883	0.0741
5	0.0957	0.0870
6	0.116	0.0851
Mean	0.0960	0.0867
SD	0.0108	0.00860
Percent CV	11.3	9.92
Percent RE	-4.00	-13.3

TABLE 5: RECOVERY OF GENTAMICIN/PAROMOMYCIN FROM HUMAN PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	(μg/ml)	PEAK HEIGHT RATIO SOLVENT	PLASMA	MEAN PERCENT RECOVERY
Gentamicin					
1	X Low	0.200	0.080	0.081	89.0
2			0.109	0.085	
3			0.111	0.100	
Mean (± SD)			0.100 ± 0.017	0.089 ± 0.010	
1	Low	0.800	0.349	0.383	102
2			0.365	0.361	
3			0.382	0.379	
Mean (± SD)			0.365 ± 0.017	0.374 ± 0.012	
1	Medium	2.50	1.140	1.005	90.6
2			1.085	1.069	
3			1.169	1.002	
Mean (± SD)			1.131 ± 0.043	1.025 ± 0.038	
1	High	5.00	2.225	2.079	94.2
2			2.306	2.237	
3			2.277	2.097	
Mean (± SD)			2.269 ± 0.041	2.138 ± 0.086	
AVERAGE =					94.0
Paromomycin					
1	X Low	0.200	0.360	0.393	91.3
2			0.356	0.301	
3			bc	0.288	
Mean (± SD)			0.358 ± 0.003	0.327 ± 0.057	
1	Low	0.800	1.330	1.416	102
2			1.380	1.417	
3			1.420	1.362	
Mean (± SD)			1.377 ± 0.045	1.398 ± 0.031	
1	Medium	2.50	4.000	3.716	90.4
2			3.990	3.922	
3			4.470	3.627	
Mean (± SD)			4.153 ± 0.274	3.755 ± 0.151	
1	High	5.00	8.290	7.399	91.5
2			8.220	8.095	
3			8.620	7.501	
Mean (± SD)			8.377 ± 0.214	7.665 ± 0.376	
AVERAGE =					93.8

bc = unacceptable chromatogram

TABLE 6A: SYSTEM STABILITY OF GENTAMICIN IN PREPARED SAMPLES

		CONCENTRATION AT ROOM TEMPERATURE STORAGE (µg/ml)			
Spiked Concentration:		0.200	0.800	2.50	5.00
TIME STORED					
0 days	Sample 1	0.192	0.826	2.62	5.26
	Sample 2	0.204	0.798	2.65	5.26
	Mean	0.198	0.812	2.64	5.26
	Percent RE	-1.00	+1.50	+5.40	+5.20
1 day	Sample 1	0.215	0.849	2.70	5.34
	Sample 2	0.232	0.804	2.67	5.26
	Mean	0.224	0.827	2.69	5.30
	Percent RE	+11.8	+3.31	+7.40	+6.00
2 days	Sample 1	0.217	0.849	2.69	5.19
	Sample 2	0.202	0.858	2.69	5.20
	Mean	0.210	0.854	2.69	5.20
	Percent RE	+4.75	+6.69	+7.60	+3.90
3 days	Sample 1	bc	0.896	2.71	5.43
	Sample 2	0.215	0.868	2.70	5.18
	Mean	0.215	0.882	2.71	5.31
	Percent RE	+7.50	+10.3	+8.20	+6.10
5 days	Sample 1	0.219	0.887	2.73	5.32
	Sample 2	0.219	0.885	2.75	5.31
	Mean	0.219	0.886	2.74	5.32
	Percent RE	+9.50	+10.8	+9.60	+6.30
6 days	Sample 1	0.230	0.909	2.80	5.42
	Sample 2	0.232	0.894	2.80	ns
	Mean	0.231	0.902	2.80	5.42
	Percent RE	+15.5	+12.7	+12.0	+8.40

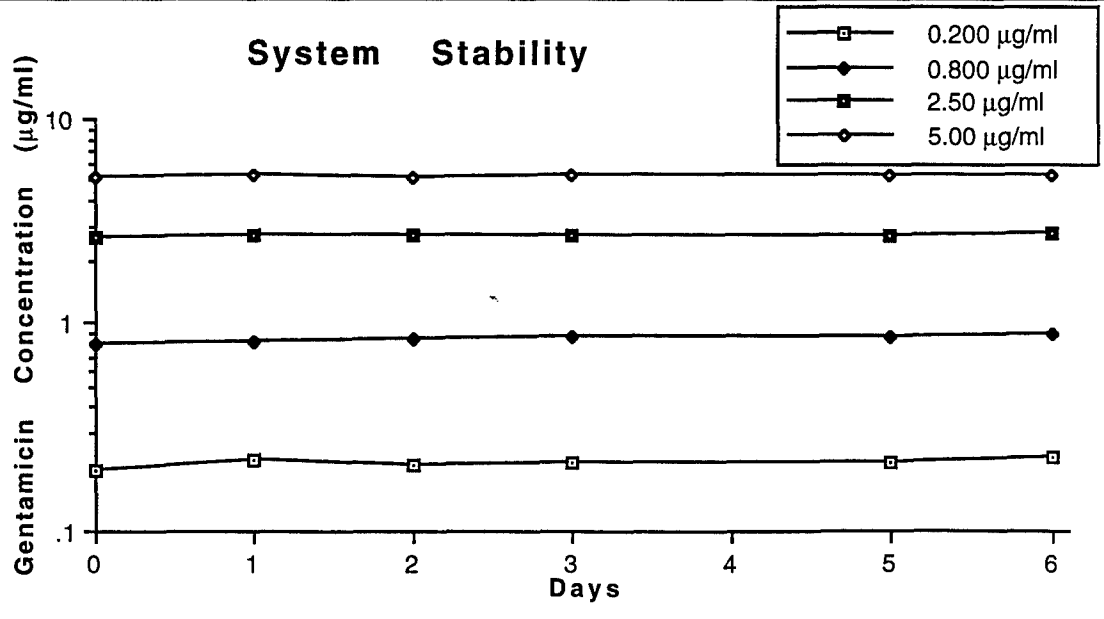


TABLE 6B: SYSTEM STABILITY OF PAROMOMYCIN IN PREPARED SAMPLES

		CONCENTRATION AT ROOM TEMPERATURE STORAGE (µg/ml)			
Spiked Concentration:		0.200	0.800	2.50	5.00
TIME STORED					
0 days	Sample 1	0.208	0.863	2.83	5.81
	Sample 2	0.202	0.791	2.55	5.64
	Mean	0.205	0.827	2.69	5.73
	Percent RE	+2.50	+3.37	+7.60	+14.5
1 day	Sample 1	0.220	0.919	2.98	5.83
	Sample 2	0.204	0.834	2.70	5.74
	Mean	0.212	0.877	2.84	5.79
	Percent RE	+6.00	+9.56	+13.6	+15.7
2 days	Sample 1	0.232	0.922	2.82	5.29
	Sample 2	0.219	0.861	2.56	5.35
	Mean	0.226	0.892	2.69	5.32
	Percent RE	+12.8	+11.4	+7.60	+6.40
3 days	Sample 1	0.209	0.824	2.64	5.23
	Sample 2	0.192	0.798	2.47	5.12
	Mean	0.201	0.811	2.56	5.18
	Percent RE	0.250	+1.37	+2.20	+3.50
5 days	Sample 1	0.173	0.773	2.47	4.96
	Sample 2	0.183	0.739	2.36	4.84
	Mean	0.178	0.756	2.42	4.90
	Percent RE	-11.0	-5.50	-3.40	-2.00
6 days	Sample 1	0.162	0.691	2.16	4.23
	Sample 2	0.169	0.683	2.16	ns
	Mean	0.166	0.687	2.16	4.23
	Percent RE	-17.3	-14.1	-13.6	-15.4

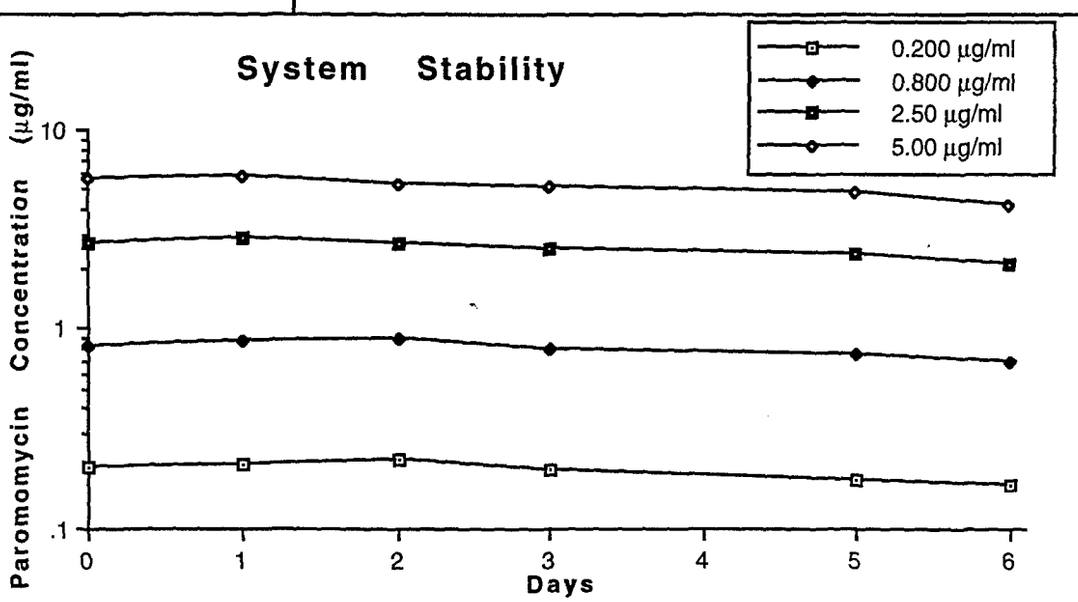


TABLE 7A: LONG TERM STABILITY OF GENTAMICIN IN HUMAN PLASMA

GENTAMICIN CONCENTRATION IN PLASMA STORED AT -20°C

Gentamicin		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.400	1.00	2.50	5.00
TIME STORED					
0 days	Sample 1	0.299	0.978	2.29	5.11
	Sample 2	0.332	0.933	2.48	4.95
	Mean	0.316	0.956	2.39	5.03
	Percent RE	-21.1	-4.45	-4.60	+0.60
1 days	Sample 1	0.400	1.00	2.46	4.89
	Sample 2	0.327	0.902	2.37	4.78
	Mean	0.364	0.951	2.42	4.84
	Percent RE	-9.12	-4.90	-3.40	-3.30
2 days	Sample 1	0.340	0.881	2.29	4.77
	Sample 2	0.313	0.905	2.25	4.60
	Mean	0.327	0.893	2.27	4.69
	Percent RE	-18.4	-10.7	-9.20	-6.30
3 days	Sample 1	0.325	0.880	2.29	4.80
	Sample 2	0.306	0.890	2.31	3.98
	Mean	0.316	0.885	2.30	4.39
	Percent RE	-21.1	-11.5	-8.00	-12.2
1 week	Sample 1	0.420	1.01	2.24	4.92
	Sample 2	0.325	0.972	2.35	4.67
	Mean	0.373	0.991	2.30	4.80
	Percent RE	-6.88	-0.90	-8.20	-4.10
2 weeks	Sample 1	0.299	0.878	2.30	4.89
	Sample 2	0.297	0.916	2.39	4.88
	Mean	0.298	0.897	2.35	4.89
	Percent RE	-25.5	-10.3	-6.20	-2.30
3 weeks	Sample 1	0.276	0.825	2.31	4.80
	Sample 2	0.285	0.816	2.13	4.52
	Mean	0.281	0.821	2.22	4.66
	Percent RE	-29.9	-18.0	-11.2	-6.80
1 month	Sample 1	0.316	0.896	2.23	4.63
	Sample 2	0.280	0.915	2.26	4.46
	Mean	0.298	0.906	2.25	4.55
	Percent RE	-25.5	-9.45	-10.2	-9.10

TABLE 7A: LONG TERM STABILITY OF GENTAMICIN IN HUMAN PLASMA

GENTAMICIN CONCENTRATION IN PLASMA STORED AT -20°C

Gentamicin		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.400	1.00	2.50	5.00
TIME STORED					
2 months	Sample 1	0.320	0.872	2.30	4.01
	Sample 2	0.328	0.883	2.08	4.06
	Mean	0.324	0.878	2.19	4.04
	Percent RE	-19.0	-12.3	-12.4	-19.3
3 months	Sample 1	0.269	0.781	1.98	3.92
	Sample 2	0.288	0.781	1.96	3.91
	Mean	0.279	0.781	1.97	3.92
	Percent RE	-30.4	-21.9	-21.2	-21.7
6 months	Sample 1	0.291	0.625	1.72	3.10
	Sample 2	0.280	0.701	1.70	3.15
	Mean	0.286	0.663	1.71	3.13
	Percent RE	-28.6	-33.7	-31.6	-37.5
1 year	Sample 1	0.272	0.560	1.38	2.68
	Sample 2	0.213	0.664	1.60	2.85
	Mean	0.243	0.612	1.49	2.77
	Percent RE	-39.4	-38.8	-40.4	-44.7

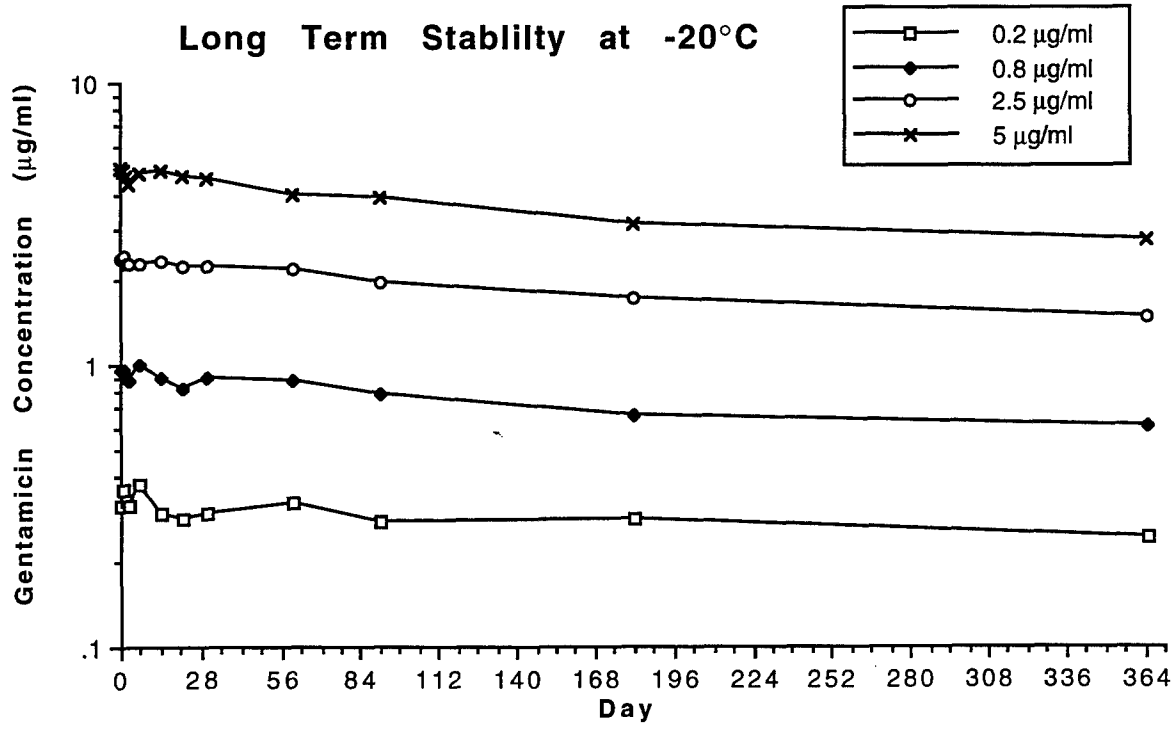


TABLE 7A: LONG TERM STABILITY OF GENTAMICIN IN HUMAN PLASMA

GENTAMICIN CONCENTRATION IN PLASMA STORED AT -70°C

		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.400	1.00	2.50	5.00
TIME STORED					
0 days	Sample 1	0.328	0.949	2.30	4.87
	Sample 2	0.323	0.940	2.40	4.96
	Mean	0.326	0.945	2.35	4.92
	Percent RE	-18.6	-5.55	-6.00	-1.70
1 day	Sample 1	0.345	0.895	2.33	4.91
	Sample 2	0.389	0.990	2.41	4.93
	Mean	0.367	0.943	2.37	4.92
	Percent RE	-8.25	-5.75	-5.20	-1.60
2 days	Sample 1	0.315	0.900	2.32	4.75
	Sample 2	0.343	0.867	2.29	4.83
	Mean	0.329	0.884	2.31	4.79
	Percent RE	-17.8	-11.7	-7.80	-4.20
3 days	Sample 1	0.246	0.845	2.32	4.78
	Sample 2	0.294	0.871	2.33	4.82
	Mean	0.270	0.858	2.33	4.80
	Percent RE	-32.5	-14.2	-7.00	-4.00
1 week	Sample 1	0.330	0.967	2.52	5.09
	Sample 2	0.310	0.925	2.41	4.74
	Mean	0.320	0.946	2.47	4.92
	Percent RE	-20.0	-5.40	-1.40	-1.70
2 weeks	Sample 1	0.400	0.916	2.32	4.86
	Sample 2	0.411	0.943	2.31	4.83
	Mean	0.406	0.930	2.32	4.85
	Percent RE	+1.37	-7.05	-7.40	-3.10
3 weeks	Sample 1	0.316	0.878	2.28	4.38
	Sample 2	0.311	0.934	2.24	4.57
	Mean	0.314	0.906	2.26	4.48
	Percent RE	-21.6	-9.40	-9.60	-10.5
1 month	Sample 1	0.310	0.862	2.25	4.68
	Sample 2	0.320	0.862	2.25	4.68
	Mean	0.315	0.862	2.25	4.68
	Percent RE	-21.3	-13.8	-10.0	-6.40

TABLE 7A: LONG TERM STABILITY OF GENTAMICIN IN HUMAN PLASMA

GENTAMICIN CONCENTRATION IN PLASMA STORED AT -70°C

		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.400	1.00	2.50	5.00
TIME STORED					
2 months	Sample 1	0.298	0.861	2.69	4.79
	Sample 2	0.306	0.835	2.24	4.55
	Mean	0.302	0.848	2.47	4.67
	Percent RE	-24.5	-15.2	-1.40	-6.60
3 months	Sample 1	0.315	0.892	2.35	4.91
	Sample 2	0.317	0.914	2.36	4.82
	Mean	0.316	0.903	2.36	4.87
	Percent RE	-21.0	-9.70	-5.80	-2.70
6 months	Sample 1	0.412	1.05	2.45	4.89
	Sample 2	0.384	1.03	2.47	4.85
	Mean	0.398	1.04	2.46	4.87
	Percent RE	-0.50	+4.00	-1.60	-2.60
1 year	Sample 1	0.356	0.959	2.25	4.59
	Sample 2	0.331	0.902	2.31	4.68
	Mean	0.344	0.931	2.28	4.64
	Percent RE	-14.1	-6.95	-8.80	-7.30

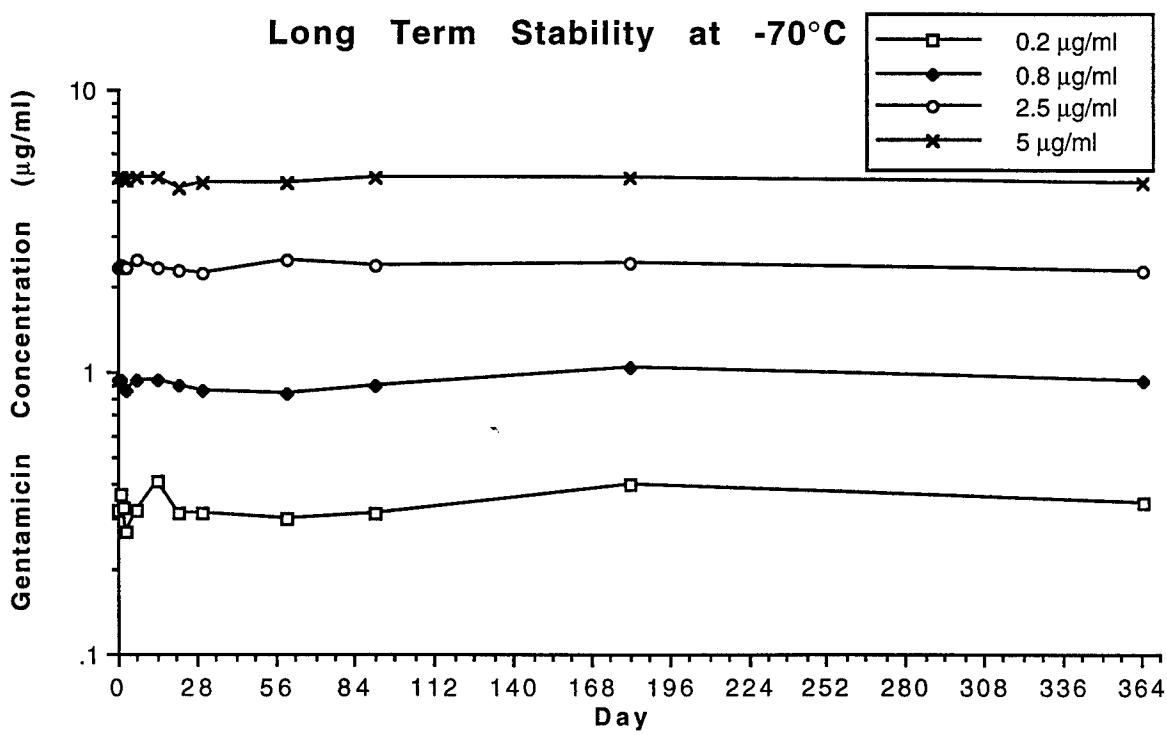


TABLE 7B: LONG TERM STABILITY OF PAROMOMYCIN IN HUMAN PLASMA

PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT -20°C

Paromomycin		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.400	1.00	2.50	5.00
TIME STORED					
0 days	Sample 1	0.365	1.07	2.41	5.16
	Sample 2	0.456	1.16	2.85	5.40
	Mean	0.411	1.12	2.63	5.28
	Percent RE	+2.62	+11.5	+5.20	+5.60
1 day	Sample 1	0.457	1.20	2.95	5.70
	Sample 2	0.410	1.03	2.60	5.00
	Mean	0.434	1.12	2.78	5.35
	Percent RE	+8.37	+11.5	+11.0	+7.00
2 days	Sample 1	0.438	1.09	2.77	5.67
	Sample 2	0.377	0.992	2.39	4.77
	Mean	0.408	1.04	2.58	5.22
	Percent RE	+1.87	+4.10	+3.20	+4.40
3 days	Sample 1	0.389	1.04	2.55	5.17
	Sample 2	0.385	1.02	2.51	4.23
	Mean	0.387	1.03	2.53	4.70
	Percent RE	-3.25	+3.00	+1.20	-6.00
1 week	Sample 1	0.430	1.21	3.08	6.67
	Sample 2	0.440	1.17	3.24	6.34
	Mean	0.435	1.19	3.16	6.51
	Percent RE	+8.75	+19.0	+26.4	+30.1
2 weeks	Sample 1	0.415	1.15	2.54	5.58
	Sample 2	0.411	1.09	2.73	5.62
	Mean	0.413	1.12	2.64	5.60
	Percent RE	+3.25	+12.0	+5.40	+12.0
3 weeks	Sample 1	0.304	0.763	2.22	3.68
	Sample 2	0.326	0.782	1.85	4.10
	Mean	0.315	0.773	2.04	3.89
	Percent RE	-21.3	-22.8	-18.6	-22.2
1 month	Sample 1	0.388	1.07	2.54	5.15
	Sample 2	0.337	1.06	2.53	4.66
	Mean	0.363	1.07	2.54	4.91
	Percent RE	-9.37	+6.50	+1.40	-1.90

TABLE 7B: LONG TERM STABILITY OF PAROMOMYCIN IN HUMAN PLASMA

PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT -20°C

Paromomycin		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.400	1.00	2.50	5.00
TIME STORED					
2 months	Sample 1	0.327	0.701	1.96	3.34
	Sample 2	0.325	0.811	1.77	3.42
	Mean	0.326	0.756	1.87	3.38
	Percent RE	-18.5	-24.4	-25.4	-32.4
3 months	Sample 1	0.265	0.675	1.57	2.93
	Sample 2	0.292	0.659	1.57	2.98
	Mean	0.279	0.667	1.57	2.96
	Percent RE	-30.4	-33.3	-37.2	-40.9
6 months	Sample 1	0.326	0.627	1.78	2.85
	Sample 2	0.334	0.668	1.66	3.20
	Mean	0.330	0.648	1.72	3.03
	Percent RE	-17.5	-35.3	-31.2	-39.5
1 year	Sample 1	0.228	0.471	1.10	1.67
	Sample 2	0.181	0.496	1.31	2.15
	Mean	0.205	0.484	1.21	1.91
	Percent RE	-48.9	-51.7	-51.8	-61.8

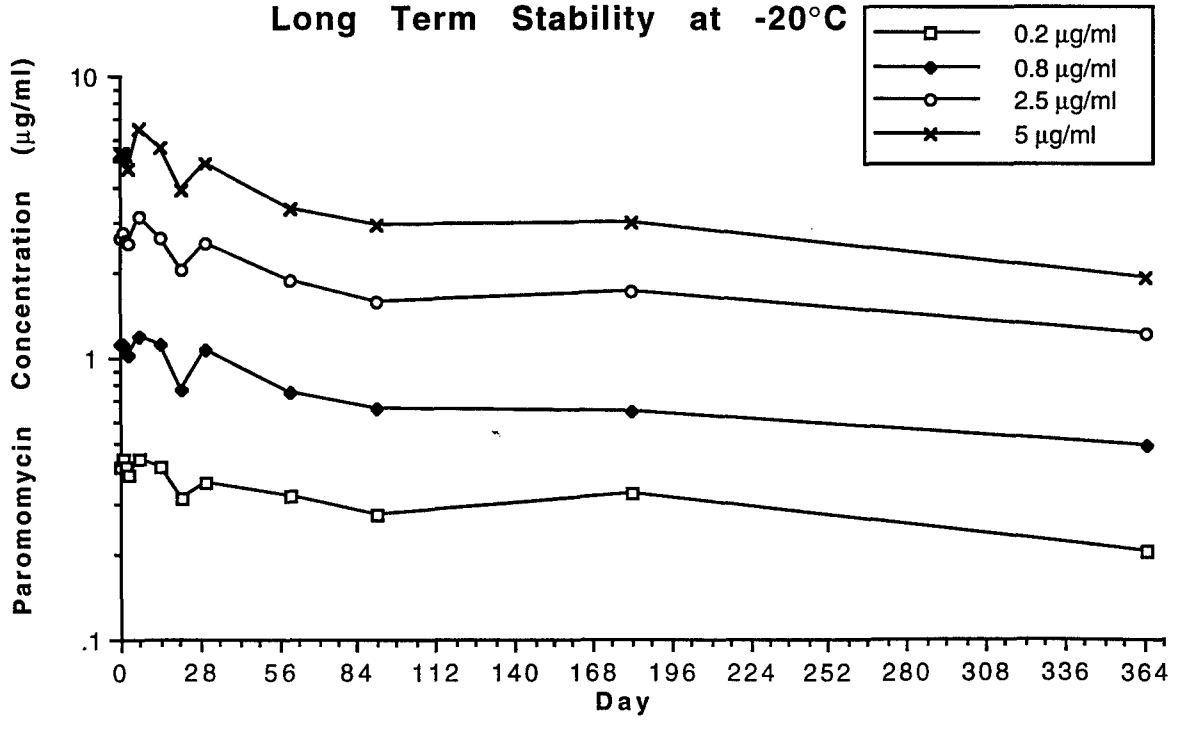


TABLE 7B: LONG TERM STABILITY OF PAROMOMYCIN IN HUMAN PLASMA

PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT -70°C

Paromomycin		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.400	1.00	2.50	5.00
TIME STORED					
0 days	Sample 1	0.415	1.07	2.45	5.03
	Sample 2	0.404	1.07	2.63	5.18
	Mean	0.410	1.07	2.54	5.11
	Percent RE	+2.37	+7.00	+1.60	+2.10
1 day	Sample 1	0.474	1.26	3.06	6.46
	Sample 2	0.482	1.22	2.96	5.96
	Mean	0.478	1.24	3.01	6.21
	Percent RE	+19.5	+24.0	+20.4	+24.2
2 days	Sample 1	0.589	1.45	3.47	6.98
	Sample 2	0.528	1.34	3.36	6.74
	Mean	0.559	1.40	3.42	6.86
	Percent RE	+39.6	+39.5	+36.6	+37.2
3 days	Sample 1	0.414	1.11	2.83	5.76
	Sample 2	0.420	1.08	2.72	5.52
	Mean	0.417	1.10	2.78	5.64
	Percent RE	+4.25	+9.50	+11.0	+12.8
1 week	Sample 1	0.484	1.39	3.62	7.19
	Sample 2	0.447	1.34	3.49	6.58
	Mean	0.466	1.37	3.56	6.89
	Percent RE	+16.4	+36.5	+42.2	+37.7
2 weeks	Sample 1	0.413	1.09	2.68	5.59
	Sample 2	0.423	1.06	2.65	5.45
	Mean	0.418	1.08	2.67	5.52
	Percent RE	+4.50	+7.50	+6.60	+10.4
3 weeks	Sample 1	0.377	0.972	2.40	4.44
	Sample 2	0.377	1.01	2.37	4.65
	Mean	0.377	0.991	2.39	4.55
	Percent RE	-5.75	-0.90	-4.60	-9.10
1 month	Sample 1	0.361	0.953	2.43	5.13
	Sample 2	0.370	0.983	2.40	4.94
	Mean	0.366	0.968	2.42	5.04
	Percent RE	-8.63	-3.20	-3.40	+0.70

TABLE 7B: LONG TERM STABILITY OF PAROMOMYCIN IN HUMAN PLASMA

PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT -70°C

Paromomycin		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.400	1.00	2.50	5.00
TIME STORED					
2 months	Sample 1	0.334	0.932	3.04	5.39
	Sample 2	0.342	0.933	2.53	5.05
	Mean	0.338	0.933	2.79	5.22
	Percent RE	-15.5	-6.75	+11.4	+4.40
3 months	Sample 1	0.390	0.991	2.52	5.07
	Sample 2	0.362	1.01	2.49	4.96
	Mean	0.376	1.00	2.51	5.02
	Percent RE	-6.00	+0.05	+0.20	+0.30
6 months	Sample 1	0.315	0.951	2.35	4.87
	Sample 2	0.309	0.918	2.36	4.75
	Mean	0.312	0.935	2.36	4.81
	Percent RE	-22.0	-6.55	-5.80	-3.80
1 year	Sample 1	0.376	1.05	2.70	5.59
	Sample 2	0.378	1.02	2.72	5.60
	Mean	0.377	1.04	2.71	5.60
	Percent RE	-5.8	+3.50	+8.4	+11.9

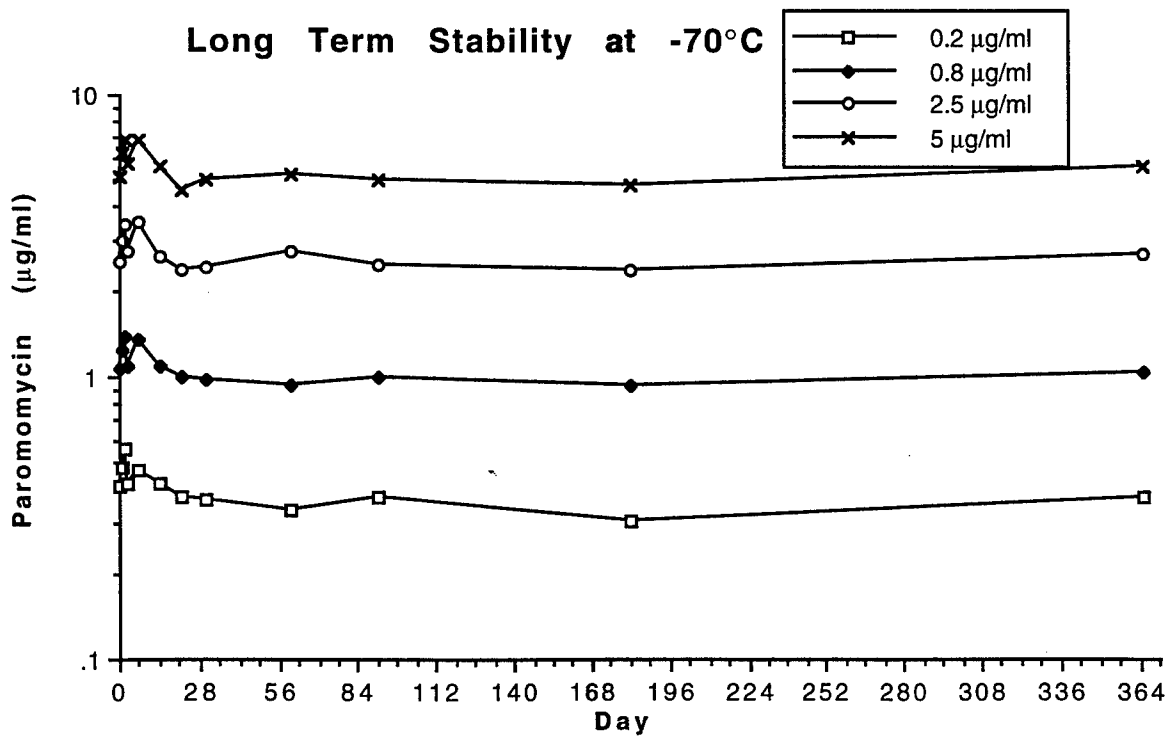


TABLE 8A: BENCH TOP STABILITY OF GENTAMICIN IN SPIKED HUMAN PLASMA

GENTAMICIN CONCENTRATION IN PLASMA STORED AT ROOM TEMPERATURE

		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.200	0.800	2.50	5.00
TIME STORED					
0 hours	Sample 1	0.219	0.798	2.56	5.01
	Sample 2	0.190	0.843	2.69	4.84
	Mean	0.205	0.821	2.63	4.93
	Percent RE	+2.50	+2.62	+5.20	-1.40
2 hours	Sample 1	0.196	0.758	2.43	5.01
	Sample 2	0.219	0.805	2.71	5.39
	Mean	0.208	0.782	2.57	5.20
	Percent RE	4.00	-2.25	+2.80	+4.00
4 hours	Sample 1	0.190	0.776	2.72	5.41
	Sample 2	0.266	0.789	2.67	5.10
	Mean	0.228	0.783	2.70	5.26
	Percent RE	14.0	-2.13	+8.00	+5.20
6 hours	Sample 1	0.168	0.751	2.55	5.14
	Sample 2	0.228	0.954	2.71	5.29
	Mean	0.198	0.853	2.63	5.22
	Percent RE	-1.00	+6.62	+5.20	+4.40

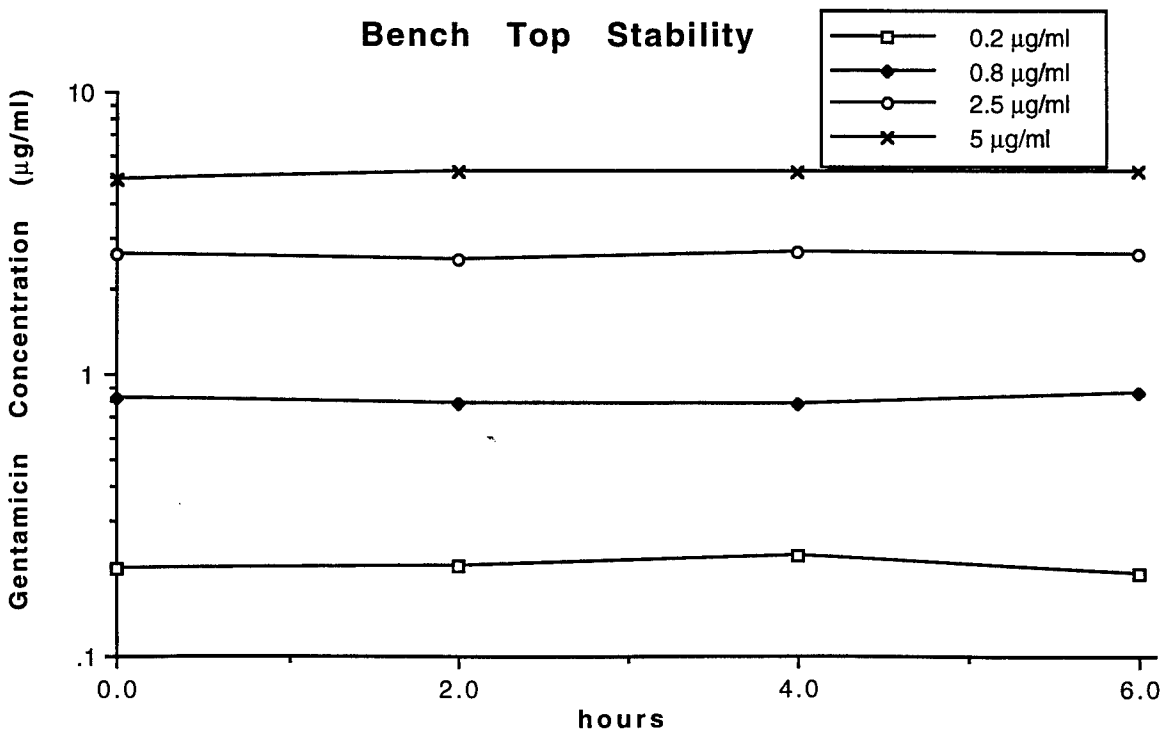


TABLE 8B: BENCH TOP STABILITY OF PAROMOMYCIN IN SPIKED HUMAN PLASMA

PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT ROOM TEMPERATURE

		CONCENTRATION (µg/ml)			
Spiked Concentration:		0.200	0.800	2.50	5.00
TIME STORED					
0 hours	Sample 1	0.232	0.910	2.91	5.67
	Sample 2	0.171	0.858	2.66	4.85
	Mean	0.202	0.884	2.79	5.26
	Percent RE	+1.00	+10.5	+11.6	+5.20
2 hours	Sample 1	0.182	0.791	2.74	5.62
	Sample 2	0.229	0.929	2.66	5.36
	Mean	0.206	0.860	2.70	5.49
	Percent RE	+3.00	+7.50	+8.00	+9.80
4 hours	Sample 1	0.179	0.814	2.73	5.48
	Sample 2	0.285	0.929	3.12	5.92
	Mean	0.232	0.872	2.93	5.70
	Percent RE	+16.0	+9.00	+17.2	+14.0
6 hours	Sample 1	0.171	0.764	2.50	5.05
	Sample 2	0.245	1.10	3.18	6.15
	Mean	0.208	0.932	2.84	5.60
	Percent RE	+4.00	+16.5	+13.6	+12.0

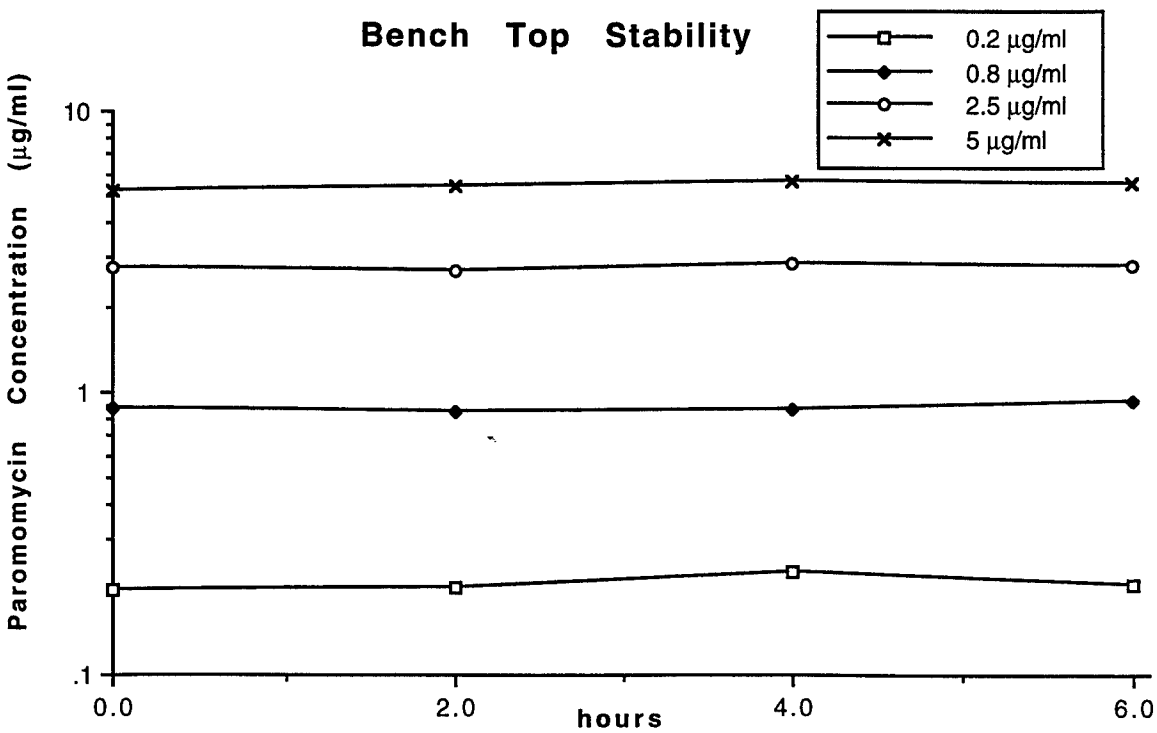


TABLE 9A: EFFECT OF REPEATED FREEZE (-70°C) AND THAW CYCLES ON GENTAMICIN SPIKED HUMAN PLASMA

Gentamicin

		CONCENTRATION (µg/ml)	
Spiked Concentration:		0.800	5.00
CYCLE			
1	Sample 1	0.891	5.12
	Sample 2	0.823	4.98
	Mean	0.857	5.05
	Percent RE	7.12	+1.00
2	Sample 1	0.808	5.28
	Sample 2	0.763	4.67
	Mean	0.786	4.98
	Percent RE	-1.75	-0.400
3	Sample 1	0.750	5.29
	Sample 2	0.695	5.12
	Mean	0.723	5.21
	Percent RE	-9.63	+4.20
4	Sample 1	0.727	5.18
	Sample 2	0.669	5.90
	Mean	0.698	5.54
	Percent RE	-12.8	+10.8
5	Sample 1	0.616	5.20
	Sample 2	0.684	4.79
	Mean	0.650	5.00
	Percent RE	-18.8	0

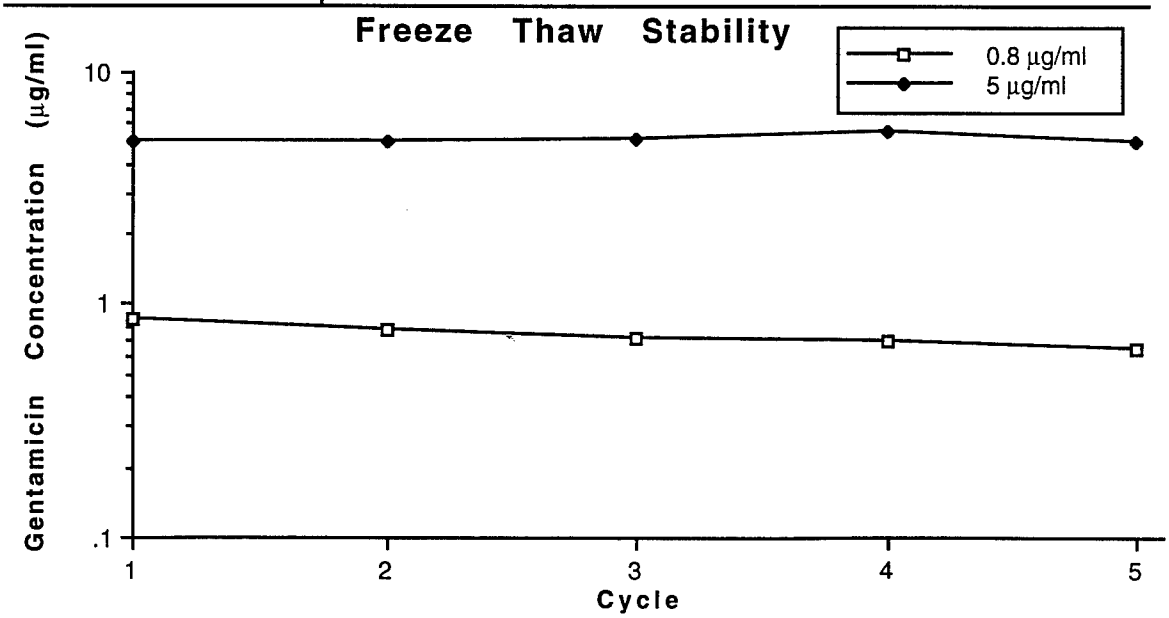


TABLE 9B: EFFECT OF REPEATED FREEZE (-70°C) AND THAW CYCLES ON PAROMOMYCIN SPIKED HUMAN PLASMA

Paromomycin

		CONCENTRATION (µg/ml)	
Spiked Concentration:		0.800	5.00
CYCLE			
1	Sample 1	0.855	4.94
	Sample 2	0.758	4.99
	Mean	0.807	4.97
	Percent RE	+0.875	-0.600
2	Sample 1	0.714	5.12
	Sample 2	0.701	4.57
	Mean	0.708	4.85
	Percent RE	-11.5	-3.00
3	Sample 1	0.658	5.51
	Sample 2	0.639	4.96
	Mean	0.649	5.24
	Percent RE	-18.9	+4.80
4	Sample 1	0.684	4.98
	Sample 2	0.651	5.76
	Mean	0.668	5.37
	Percent RE	-16.5	+7.40
5	Sample 1	0.639	5.32
	Sample 2	0.681	4.87
	Mean	0.660	5.10
	Percent RE	-17.5	+2.00

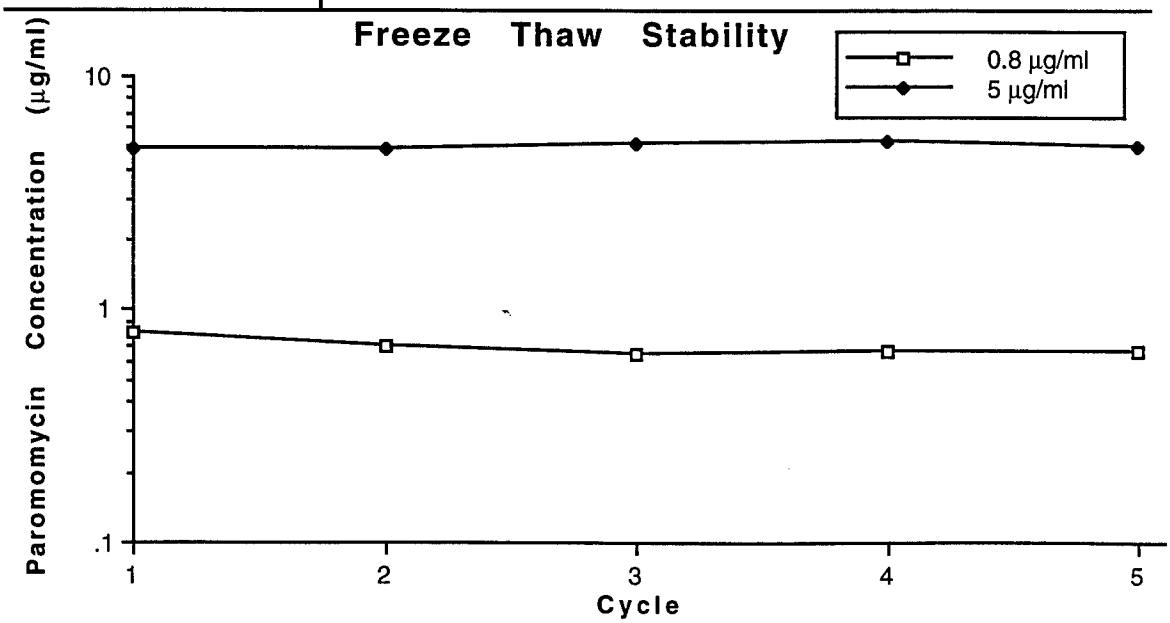


TABLE 10A: ACCURACY OF GENTAMICIN HUMAN PLASMA ASSAY (BLIND STUDY RESULTS)

Gentamicin free base

Sample Number	Spiked Level (µg/ml)	Measured Level# (µg/ml)	Statistics (µg/ml)	
2	0	0		
9		0		
16		0		
17		0		
20		0		
24		0		
8	0.2	0.211	Mean =	0.199
12		0.203	SD =	0.00844
13		0.200	Percent CV =	4.23
15		0.194	Percent Bias =	-0.3
18		0.189		
3	0.4	0.385	Mean =	0.402
7		0.378	SD =	0.0336
11		0.394	Percent CV =	8.35
22		0.461	Percent Bias =	0.5
23		0.392		
1	1	0.831	Mean =	0.806
4		0.774	SD =	0.027
10		0.834	Percent CV =	3.35
21		0.784	Percent Bias =	-19.4
26		0.806		
5	8	5.86	Mean =	5.93
6		5.72	SD =	0.28
14		5.63	Percent CV =	4.73
19		6.25	Percent Bias =	-25.9
25		6.20		

Measured concentrations are averages of three analyses.

TABLE 10B: ACCURACY OF PAROMOMYCIN HUMAN PLASMA ASSAY (BLIND STUDY RESULTS)

Paromomycin free base

Sample Number	Spiked Level (µg/ml)	Measured Level# (µg/ml)	Statistics (µg/ml)	
5	0	0		
9		0		
10		0		
13		0		
22		0		
26		0		
3	0.2	0.215	Mean =	0.205
14		0.177	SD =	0.0167
18		0.208	Percent CV =	8.16
20		0.205	Percent Bias =	2.5
21		0.220		
6	0.5	0.557	Mean =	0.498
11		0.495	SD =	0.0337
15		0.482	Percent CV =	6.76
17		0.475	Percent Bias =	-0.36
24		0.482		
4	3	3.03	Mean =	2.96
7		2.97	SD =	0.101
12		2.94	Percent CV =	3.42
16		2.88	Percent Bias =	-1.33
25		3.06		
1	9	9.15	Mean =	8.71
2		8.65	SD =	0.258
8		8.49	Percent CV =	2.97
19		8.57	Percent Bias =	-3.27
23		8.67		

Measured concentrations are averages of three analyses.

TABLE 11: PRECISION STANDARD CURVE DATA FOR GENTAMICIN/ PAROMOMYCIN RAT PLASMA ASSAY, STUDY REPORT 24

Gentamicin Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
3/1/95	rintrage	0.44400042	-0.0050161	0.99988014
3/8/95	ginte3ra	0.45454769	0.00537975	0.99948715
3/9/95	rtinte3g	0.42375694	0.01053217	0.99970313

Gentamicin Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml)							
	0.100	0.200	0.400	0.800	1.50	3.00	6.00	12.0
	Back Calculated Concentration (µg/ml)							
rintrage	0.0946	0.205	0.426	0.788	1.5	2.98	6.02	12
ginte3ra	0.1	0.197	0.391	0.842	1.46	3.1	5.86	12.1
rtinte3g	0.103	0.195	0.402	0.827	1.47	3	5.87	12.1
n 3	3	3	3	3	3	3	3	
Mean	0.0992	0.199	0.406	0.819	1.48	3.03	5.92	12.1
SD	0.00426	0.00529	0.0179	0.0279	0.0208	0.0643	0.0896	0.0577
Percent CV	4.29	2.66	4.4	3.4	1.41	2.12	1.51	0.478
Percent RE	-0.8	-0.5	1.58	2.37	-1.56	0.889	-1.39	0.556

Paromomycin Standard Curve Parameters

Validation Run Date	Validation Run	Slope	Intercept	Coefficient of Determination
3/1/95	rintrapa	1.54571936	0.05064803	0.99960491
3/8/95	pinte3rt	1.61517291	0.01253853	0.9988574
3/9/95	rainte3p	1.3005778	0.02468605	0.99967686

Paromomycin Back Calculated Standard Calibrators

Validation Run	Spiked Concentration (µg/ml)							
	0.100	0.200	0.400	0.800	1.50	3.00	6.00	12.0
	Back Calculated Concentration (µg/ml)							
rintrapa	0.0863	0.204	0.425	0.806	1.53	3.08	6.05	11.8
pinte3rt	0.087	0.208	0.403	0.886	1.52	3.06	5.75	12.1
rainte3p	0.104	0.186	0.389	0.86	1.52	2.95	6	12
n 3	3	3	3	3	3	3	3	
Mean	0.0924	0.199	0.406	0.851	1.52	3.03	5.93	12
SD	0.01	0.0117	0.0181	0.0408	0.00577	0.07	0.161	0.153
Percent CV	10.8	5.88	4.47	4.8	0.379	2.31	2.71	1.28
Percent RE	-7.57	-0.333	1.42	6.33	1.56	1	-1.11	-0.278

TABLE 12A: PRECISION OF GENTAMICIN RAT PLASMA ASSAY

Interday Precision Gentamicin

Validation Run	QC Sample No.	Spiked Concentrations (µg/mL) 0.200	0.800	2.50	5.00
		Measured Concentrations (µg/mL)			
rintrage	1	0.2	0.829	2.08	4.47
	2	0.191	0.811	2.29	4.64
ginte3ra	1	0.221	0.855	2.55	4.57
	2	0.199	0.87	2.61	4.75
rtinte3g	1	0.188	0.87	2.47	4.72
	2	0.221	0.803	2.63	5.07
n		6	6	6	6
Mean		0.203	0.84	2.44	4.7
SD		0.0144	0.0295	0.215	0.206
Percent CV		7.1	3.51	8.8	4.39
Percent RE		1.67	4.96	-2.47	-5.93

Intraday Precision Gentamicin

Validation Run	QC Sample No.	Spiked Concentrations (µg/mL) 0.200	0.800	2.50	5.00
		Measured Concentrations (µg/mL)			
rintrage	1	0.216	0.802	2.2	4.71
	2	0.209	0.784	2.47	4.7
	3	0.194	0.829	2.26	4.61
	4	0.203	0.804	2.28	4.62
	5	0.212	0.802	2.21	4.86
	6	0.2	0.838	2.19	4.68
	n	6	6	6	6
	Mean	0.206	0.81	2.27	4.7
	SD	0.00816	0.0199	0.105	0.09
	Percent CV	3.97	2.46	4.63	1.92
	Percent RE	2.83	1.23	-9.27	-6.07

TABLE 12B: PRECISION OF PAROMOMYCIN RAT PLASMA ASSAY

Interday Precision Paromomycin

Validation Run No.	QC Sample No.	Spiked Concentrations (µg/mL) 0.200	0.800	2.50	5.00
		Measured Concentrations (µg/mL)			
rintrapa	1	0.201	0.899	2.01	4.7
	2	0.187	0.882	2.27	4.74
pinte3rt	1	0.189	0.806	2.41	4.2
	2	0.224	0.989	2.89	4.93
rainte3p	1	0.177	0.902	2.57	4.93
	2	0.206	0.833	2.63	5.03
	n	6	6	6	6
	Mean	0.197	0.885	2.46	4.76
	SD	0.0167	0.0636	0.306	0.299
	Percent CV	8.45	7.19	12.4	6.3
	Percent RE	-1.33	10.6	-1.47	-4.9

Intraday Precision Paromomycin

Validation Run No.	QC Sample No.	Spiked Concentrations (µg/mL) 0.200	0.800	2.50	5.00
		Measured Concentrations (µg/mL)			
rintrapa	1	0.21	0.905	2.38	5.07
	2	0.214	0.934	2.84	5.25
	3	0.203	0.971	2.23	5.32
	4	0.166	0.791	2.19	4.5
	5	0.17	0.766	2.07	4.69
	6	0.144	0.746	1.93	4.3
	n	6	6	6	6
	Mean	0.185	0.852	2.27	4.86
	SD	0.0285	0.096	0.316	0.419
	Percent CV	15.4	11.3	13.9	8.64
	Percent RE	-7.75	6.52	-9.07	-2.9

TABLE 13: LOWER LIMIT OF QUANTITATION OF THE RAT PLASMA ASSAY FOR GENTAMICIN/PAROMOMYCIN

Gentamicin

Spiked Concentration	0.100 µg/ml	0.100 µg/ml
Sample	Measured Concentrations (µg/ml)	
	Interday	Intraday
1	0.0946	0.0929
2	0.1	0.0974
3	0.103	0.0861
4	-	0.0996
5	-	0.0906
6	-	0.0974
Mean	0.0992	0.094
SD	0.00426	0.00509
Percent CV	4.29	5.42
Percent RE	-0.8	-6

Paromomycin

Spiked Concentration	0.100 µg/ml	0.100 µg/ml
Sample	Measured Concentrations (µg/ml)	
	Interday	Intraday
1	0.0863	0.0834
2	0.087	0.0877
3	0.104	0.0758
4	-	0.0958
5	-	0.0839
6	-	0.0915
Mean	0.0924	0.0864
SD	0.01	0.00698
Percent CV	10.8	8.08
Percent RE	-7.57	-13.7

TABLE 14: RECOVERY OF GENTAMICIN/PAROMOMYCIN FROM RAT PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	(µg/ml)	PEAK HEIGHT RATIO SOLVENT	PLASMA	MEAN PERCENT RECOVERY
Gentamicin					
1	X Low	0.200	0.051	0.048	95.3
2			0.049	0.045	
3			0.049	0.049	
Mean (± SD)			0.050 ±0.001	0.047 ±0.002	
1	Low	0.800	0.186	0.177	96.0
2			0.178	0.176	
3			0.187	0.176	
Mean (± SD)			0.184 ±0.005	0.176 ±0.001	
1	Medium	2.50	0.576	0.523	89.3
2			0.579	0.514	
3			0.58	0.512	
Mean (± SD)			0.578 ±0.002	0.516 ±0.006	
1	High	5.00	1.137	0.998	93.2
2			1.136	1.051	
3			1.121	1.114	
Mean (± SD)			1.131 ±0.009	1.054 ±0.058	
AVERAGE =					93.4
Paromomycin					
1	X Low	0.200	0.21	0.128	69.7
2			0.198	0.136	
3			0.196	0.157	
Mean (± SD)			0.201 ±0.008	0.140 ±0.015	
1	Low	0.800	0.805	0.545	68.5
2			0.829	0.544	
3			0.789	0.571	
Mean (± SD)			0.808 ±0.020	0.553 ±0.015	
1	Medium	2.50	2.44	1.592	65.2
2			2.467	1.546	
3			2.422	1.644	
Mean (± SD)			2.443 ±0.023	1.594 ±0.049	
1	High	5.00	4.709	3.117	69.5
2			4.867	3.167	
3			4.654	3.603	
Mean (± SD)			4.743 ±0.111	3.296 ±0.267	
AVERAGE =					68.2

b.c. = unacceptable chromatogram

LABORATORY METHODOLOGY FOR PYRIDOSTIGMINE (CATION) PLASMA ASSAY,* STUDY REPORT 25

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. LC-600 Shimadzu Pump (Shimadzu Corp., Kyoto, Japan) or equivalent.
3. Shimadzu SPD 10A UV Detector (Shimadzu Corp., Kyoto, Japan) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. All solvents are HPLC grade unless otherwise specified.
2. All chemicals are reagent grade unless otherwise specified.
3. Pyridostigmine bromide, lot no. 8950426 (Sigma). The certificate of analysis lists the chemical formula as $C_9H_{13}BrN_2O_2$ and the purity as 99% by thin layer chromatography.
4. Neostigmine bromide, lot no. KT05130J (Aldrich Chemicals).
5. Tetramethylammonium chloride (TMACl) (Fluka Chemika).
6. Acetonitrile (Fisher Scientific, Fair Lawn, NJ).
7. Phosphoric acid (Fisher Scientific, Fair Lawn, NJ).
8. Water (deionized by Nanopure II, Barnstead Co., Boston, MA).
9. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).

*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

C. ASSAY CONDITIONS

1. DETECTOR

   Settings

   Wavelength; 208 nm
   Sensitivity: 0.003 aufs
   Rise Time: 1.0 s

   Lamp

   Deuterium Lamp -2900-0484, ABI Analytical, Inc., Ramsey, NJ.

2. COLUMN

   Axxiom Silica, 5 µm particle size, 4.6 x 250 mm (Richard Scientific, Novato, CA) or equivalent.

3. SOLVENT SYSTEM

   CH_3CN/H_2O (1:1, v/v) with 0.05% TMACl, 5 mM $(NH_4)_2HPO_4$ (final concentrations) apparent pH = 7.2

4. FLOW RATE

   1.0 ml/min

5. REPRESENTATIVE STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and checked for deterioration by comparison to a newly made solution (solutions are discarded when a more than 10% change in the absolute peak height is observed or by 6 months after the preparation date).

   a. Pyridostigmine bromide for interday plasma precision expressed as the cation concentration.

Prep date: 11/23/94

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent**	Conc. (µg/ml)
Standard Curve	11.74	0.694	106.75	acid water	76.3
Control	12.67	0.694	115.24	acid water	76.3

*= Molecular weights of pyridostigmine cation/pyridostigmine hydrochloride
** = Water acidified by addition of a drop of 1 N HCl.

   b. Neostigmine bromide expressed as the bromide concentration-Internal standard for interday plasma precision.

Prep date: 11/14/94

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	7.79	1	103.9	50% MeOH	75.0

6. REPRESENTATIVE WORKING SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and discarded when stock solutions were discarded or by 6 months after the preparation date).

   a. Pyridostigmine (cation) solutions.

Prep date: 11/23/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent*	Conc. (µg/ml)
Standard Curve	76.3	0.500	50	acid water	0.763
Control	76.3	0.500	50	acid water	0.763

** = Water acidified by addition of a drop of 1 N HCl.

   b. Neostigmine bromide - Internal standard for interday plasma precision.

Prep date: 9/7/94

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	75.0	1	200	50% MeOH	0.375

7. RETENTION TIMES (subject to change depending on temperature and column performance).

   a. Neostigmine bromide (Internal Standard) - 14 min
   b. Pyridostigmine (cation) - 16 min

8. BLANK PLASMA AND BLOOD

   Human plasma (CPD or CPDA-1 as anticoagulant) is obtained from the San Francisco Irwin Memorial Blood Bank.

9. INJECTION VOLUME

   25-50 µl - Samples that are expected to have high pyridostigmine concentrations (i.e. high standard curve calibrators, high concentration control samples, and sponsor samples shown in early runs to be near C_{peak} or in later runs expected to be near C_{peak}) are injected at the low end of the volume range.

10. BOND ELUT CARTRIDGES

    C8 Bond Elut (500 mg packing, Varian, Harbor City, CA). New C8 Bond Elut cartridges were prepared by washing (fill up the columns) with CH_3CN and water.

11. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves are calculated by weighted linear regression where weights = 1/y.

12. LOWER LIMIT OF QUANTITATION OF METHOD (The minimum pyridostigmine (cation) quantitation limit for the assay of human plasma was based on the interday and intraday low point validation results, on standard curve calibrator results, and a minimum 3 to 1 signal to noise ratio.)

    1.53 ng/ml pyridostigmine (cation) in plasma.

13. VOLUME MEASUREMENT

    Plasma sample volumes were measured with a 200 µl or a 1000 µl Gilson Pipetman. Blood sample volumes were measured with Eppendorf pipettes. Hamilton syringes were used to measure standard and control solution volumes.

14. WISP OPERATING TEMPERATURE

    Room temperature.

15. SATURATING THE MOBILE PHASE WITH SILICA

    The mobile phase is recycled through a non analytical silica gel column overnight to saturate it with silica.

16. SAMPLE EVAPORATION

    Extracted samples are evaporated in a N-EVAP® Model 112 (Organomatic Assoc, Inc., S.Berlin, MA) by passing N_2 over the sample. The samples do not sit in water during evaporation.

D. SAMPLE STORAGE

All samples were kept frozen at -70°C before analysis and thawed for preparation and analysis, unless specified otherwise.

E. SAMPLE PREPARATION

1. Vortex frozen specimens for 20 seconds after sample thaws.

2. Pipet 0.5 ml of a plasma sample into a clean glass culture tube (13 x 100).

3. Spike standard curve samples as shown in Section G "Generation of Standard Curve Calibrators" and vortex for 10 s.

4. Add 50 µl of internal standard (0.375 µg/ml neostigmine bromide) solution.

5. Vortex for 10 seconds.

6. Add 1 ml CH_3CN to precipitate plasma proteins.

7. Vortex 1 min.

8. Centrifuge 10 minutes at 3000 *g*.

9. Pour supernatant into a prewashed C8 Bond Elut cartridge (500 mg) and successively wash with 2 ml H_2O, 4 ml CH_3CN/H_20 (50:50), 2 ml CH_3CN, and 0.5 ml CH_3CN/H_2O (85:15) containing 1.0 mM $(NH_4)_2HPO_4$ at pH 3.6. Discard washings. Elutions from Bond Elut cartridges were performed using the gravity drip method.

10. Elute with 2 ml CH_3CN/H_2O (85:15) containing 1.0 mM $(NH_4)_2HPO_4$ at pH 3.6.

11. Evaporate to approximately 200 µl under N_2 at room temperature.

12. Transfer to WISP vial and inject onto HPLC column.

F. QUALITY CONTROL

1. Content and frequency of blanks

   A blank plasma sample was prepared as described in "Sample Preparation" and assayed at least once for each standard curve in precision assays.

2. PIPETTE CALIBRATION

   See SOP 2C-1.1.

3. BALANCE CALIBRATION

   See SOP 2C-2.1

G. GENERATION OF STANDARD CURVE CALIBRATORS

A representative example of the generation of standard curve calibrators is shown in the table below. Spike blank plasma standard curve samples with pyridostigmine (cation) solution to make a standard curve. This procedure is equivalent to addition of the

masses of pyridostigmine (cation) shown below. Since 0.500 ml plasma samples are assayed, these amounts correspond to the nominal cation concentrations shown below. Vortex for 10s.

Generation of Pyridostigmine (cation)
Standard Curve Samples

Sample	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Mass Spiked (ng)	Standard Curve Sample Nominal Concentration (ng/ml)
00*	0	0	0	0
0**	0	0	0	0
1	1	0.763	0.763	1.53
2	2	0.763	1.526	3.05
3	4	0.763	3.052	6.10
4	8	0.763	6.104	12.2
5	15	0.763	11.45	22.9
6	25	0.763	19.08	38.2
7	40	0.763	30.52	61.0
8	50	0.763	38.15	76.3

H. GENERATION OF PRECISION SAMPLES

A representative example of the generation of precision controls is shown in the table below. Samples for precision analysis were prepared by spiking 0.5 ml plasma specimens with control working solutions to make the pyridostigmine (cation) concentrations shown.

Generation of Pyridostigmine Precision Samples

	Volume Spiked (µl)	Spiking Solution Concentration (µg/ml)	Control Volume (ml)	Precision Sample Nominal Concentration (ng/ml)
X-Lo	2	0.763	0.5	3.05
Low	6	0.763	0.5	9.16
Med.	20	0.763	0.5	30.5
Hi	30	0.763	0.5	45.8

I. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at four different concentrations by comparing the pyridostigmine (cation) to internal standard peak height ratios in reference samples to the peak height ratios in plasma. Plasma (0.5 ml) samples were spiked with pyridostigmine (cation) then prepared as described above in "Sample Preparation," except the internal standard was added after the elution (step 10). Reference samples were generated by preparing 0.5 ml plasma as described above in "Sample Preparation," except that pyridostigmine was spiked and internal standard added after the elution.

*00 = Sample with no drug and no internal standard.

**0 = Sample with no drug but with internal standard.

J. GENERATION OF STABILITY SAMPLES

System stability and bench top stability samples were generated in the same way as precision control samples.

The effect of repeated freeze and thaw cycles on stabilities of pyridostigmine (cation) in human plasma samples was determined as follows: Spiked (low and high concentrations) pooled biological samples were subjected to five thaw/freeze cycles. For each cycle, a duplicate set of thaw/freeze samples (0.5 ml) was generated at each concentration. The study is run with the following procedure:

a. Prepare high and low concentration samples labeled H-1, H-2 ... H-5, and L-1, L-2 ... L-5, in duplicate.

b. Store all samples until frozen at the specified temperature.

c. Repeatedly thaw and refreeze samples according to the following table. Thaw as if for sample preparation to room temperature. Let thawed samples stand at room temperature for 1 h.

Cycle	Keep these samples in freezer	Thaw these samples
1	1	2, 3, 4, 5
2	1, 2	3, 4, 5
3	1, 2, 3	4, 5
4	1, 2, 3, 4	5
5	1, 2, 3, 4, 5	none

d. Following Cycle 5, take out all of the samples, thaw to room temperature, and assay the samples with a standard curve.

K. VALIDATION RESULTS

1. STANDARD CURVE

Chromatograms for each point in representative standard curves for pyridostigmine (cation) appear in Figure 3. Peak height ratios for these calibrators appear in Table 1. Statistical parameters of plasma interday precision standard curve calibrators appear in Table 2.

2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 3A-B.

3. LLOQ
   Results for this evaluation appear in Table 4.

4. RECOVERY
   Results for this evaluation appear in Table 5.

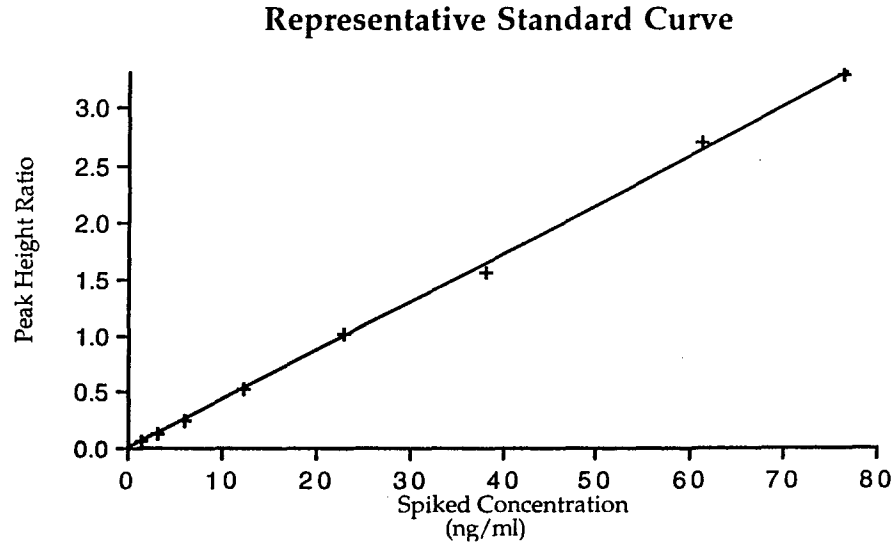
5. STABILITY
   a. System Stability: Results appear in Table 6.
   b. Long Term Stability: Results appear in Table 7.
   c. Bench Top Stability: Results appear in Table 8.
   d. Freeze/Thaw Stability: Results appear in Table 9.

6. BLIND SAMPLE ANALYSIS
   Results appear in Table 10.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR PYRIDOSTIGMINE (FREE BASE) HUMAN PLASMA ASSAY, STUDY REPORT 25

SPIKED AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	-	-
0.7630	1.53	0.061	1.41
1.526	3.05	0.145	3.36
3.052	6.10	0.259	6.01
6.104	12.2	0.527	12.2
11.45	22.9	1.022	23.7
19.08	38.2	1.569	36.4
30.52	61.0	2.699	62.6
38.15	76.3	3.268	75.8

Regression equation:***
$y = 0.0431x + 0.00002$, $r^2 = 0.9988$



* Into 0.5 ml of biological sample.
** Ratio of drug peak height to internal standard peak height.
*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 2: PRECISION STANDARD CURVE DATA FOR PYRIDOSTIGMINE HUMAN PLASMA ASSAY, SR 25

Pyridostigmine Cation Standard Curve Parameters

Validation Run Date	Validation Run No.	Slope	Intercept	Coefficient of Determination
11/28/94	1	0.03895819	0.02074876	0.99767455
12/2/94	2	0.03970787	0.01593752	0.99814691
12/3/94	3	0.0431179	2.3361E-05	0.9988433
12/2/94	4	0.04117508	-0.0084501	0.99885733
12/4/94	5	0.04069309	0.0099219	0.99693227
12/6/94	6	0.04602067	0.0217952	0.9960465
12/20/94	7	0.04662032	0.00526143	0.99715104

Pyridostigmine Cation Back Calculated Standard Calibrators

Run Number	Spiked Concentration (ng/mL)							
	1.53	3.05	6.1	12.2	22.9	38.2	61	76.3
	Back Calculated Concentration (ng/mL)							
1	1.24	3.63	5.94	12.9	24.2	35.9	61.9	76.0
2	1.69	3.00	6.30	11.4	21.7	38.2	62.9	bc
3	1.41	3.36	6.01	12.2	23.7	36.4	62.6	75.8
4	1.37	3.17	6.50	13.3	21.9	38.0	60.4	76.7
5	1.60	3.47	5.78	12.1	20.8	36.3	62.6	79.1
6	1.29	bc	6.35	13.1	24.3	40.1	56.4	77.3
7	1.47	3.58	6.15	11.7	21.5	35.8	62.9	78.7
n	7	6	7	7	7	7	7	6
Mean	1.44	3.37	6.15	12.4	22.6	37.2	61.4	77.3
S.D.	0.162	0.244	0.253	0.727	1.44	1.59	2.37	1.38
Percent CV	11.3	7.26	4.12	5.87	6.37	4.26	3.86	1.78
Percent Rel. Err.	-5.98	10.4	0.773	1.52	-1.37	-2.51	0.632	1.27

bc = unacceptable chromatogram

TABLE 3: PRECISION OF PYRIDOSTIGMINE (CATION) HUMAN PLASMA ASSAY

Inter-Run Precision Pyridostigmine Cation

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 3.05	9.16	30.5	45.8
		Measured Concentrations (ng/mL)			
1	1	3.73	9.86	30.7	51.3
	2	3.34	9.79	31.8	51.4
2	1	3.10	11.7	30.4	41.3
	2	2.90	12.3	29.4	47.0
3	1	3.22	8.16	31.8	48.9
	2	3.34	9.49	30.3	47.0
4	1	3.61	9.17	29.4	33.3
	2	2.76	7.32	30.0	41.6
5	1	3.27	9.71	29.5	49.7
	2	bc	9.09	29.6	47.7
6	1	2.66	6.54	32.1	46.6
	2	2.81	9.54	31.0	43.2
n		11	12	12	12
Mean		3.16	9.39	30.5	45.8
S.D.		0.348	1.61	0.989	5.17
Percent CV		11.0	17.1	3.24	11.3
Percent R.E.		3.55	2.50	0	-0.109

Intra-Run Precision Pyridostigmine Cation

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 3.05	9.16	30.5	45.8
		Measured Concentrations (ng/mL)			
7	1	2.80	9.15	28.7	39.5
	2	2.53	8.85	29.1	41.6
	3	3.21	8.51	29.7	42.8
	4	3.25	8.92	28.9	42.9
	5	3.25	9.48	29.6	46.5
	6	3.00	9.50	31.2	44.5
Mean		3.01	9.07	29.5	43.0
S.D.		0.293	0.386	0.905	2.398
Percent CV		9.75	4.25	3.06	5.58
Percent R.E.		-1.42	-1.00	-3.17	-6.19

TABLE 4: LOWER LIMIT OF QUANTITATION OF THE HUMAN PLASMA ASSAY FOR PYRIDOSTIGMINE BASE

Spiked Concentration	1.53 ng/ml	1.53 ng/ml
Sample	Measured Concentrations (ng/ml)	
	Interday	Intraday
1	1.24	1.88
2	1.69	1.55
3	1.41	1.57
4	1.37	1.34
5	1.60	1.96
6	1.29	1.50
Mean	1.43	1.63
Standard Deviation	0.18	0.24
Percent CV	12.3	14.6
Percent Error	-6.32	6.72

TABLE 5: RECOVERY OF PYRIDOSTIGMINE FROM HUMAN PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	(ng/ml)	PEAK HEIGHT RATIO SOLVENT	PLASMA	MEAN PERCENT RECOVERY
1	X Low		0.137	0.080	62.7
2			0.121	0.080	
3			0.131	0.084	
Mean (± SD)		3.05	0.130 ±0.008	0.081 ±0.002	
1	Low		0.355	0.239	67.6
2			0.331	0.225	
3			0.344	0.232	
Mean (± SD)		9.16	0.343 ±0.012	0.232 ±0.007	
1	Medium		1.096	0.763	69.1
2			1.126	0.770	
3			1.102	0.764	
Mean (± SD)		30.5	1.108 ±0.016	0.766 ±0.004	
1	High		1.717	1.175	67.9
2			1.682	1.156	
3			1.695	1.127	
Mean (± SD)		45.8	1.698 ±0.018	1.153 ±0.024	
AVERAGE =					66.8

TABLE 6: SYSTEM STABILITY IN PREPARED HUMAN PLASMA

Concentration for Prepared Biological Samples Stored at Room Temperature

Pyridostigmine (Cation)

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	3.05	9.16	30.5	45.8
TIME STORED				
0 day	2.74	8.04	31.6	44.9
1 day	2.85	8.79	29.6	46.9
2 day	2.28	9.00	29.8	43.9
3 day	2.94	8.37	30.2	44.0
4 day	2.58	8.85	29.8	45.5
5 day	2.47	9.02	30.0	45.8
6 day	2.55	8.75	29.7	45.5

* Measured concentrations are averages of two analyses.

TABLE 7: FREEZER STABILITY OF PYRIDOSTIGMINE (CATION) IN HUMAN PLASMA#

PYRIDOSTIGMINE (CATION) CONCENTRATION IN PLASMA STORED AT -80°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	3.43	10.3	27.3	47.8
DAYS STORED				
0	4.25	11.1	26.6	43.5
1	3.16	10.4	25.8	46.0
2	3.57	10.4	24.4	46.6
3	3.70	10.2	26.6	47.8
6	2.81	9.60	26.9	29.6
13	3.77	9.88	24.5	44.9
20	3.50	10.4	24.8	44.3
29	3.16	10.0	28.1	48.4
57	4.08	12.6	29.8	49.3
99	2.54	8.64	26.2	43.8
135	4.21	10.8	29.3	45.3
MEAN ±SD	3.52 ±0.56	10.4 ±0.98	26.6 ±1.82	46.3 ±2.18

PYRIDOSTIGMINE (CATION) CONCENTRATION IN PLASMA STORED AT -20°C

	CONCENTRATION (ng/ml)			
Spiked Concentration:	3.43	10.3	27.3	47.8
DAYS STORED				
0	3.16	9.47	22.4	42.2
1	3.16	9.75	28.1	47.2
3	3.50	9.95	28.9	44.3
6	4.66	9.95	28.8	42.9
13	2.61	10.1	25.2	37.5
20	3.16	9.19	23.1	35.7
57	2.32	6.72	18.7	39.7
99	0.75	4.32	6.79	16.2
135	1.87	3.90	6.08	15.8

Data obtained according to the method described in Study Report No. 3, dated Jan. 22, 1985 and titled "High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma."
* Measured concentrations are averages of two analyses.

TABLE 8: BENCH TOP STABILITY OF PYRIDOSTIGMINE (CATION) IN SPIKED HUMAN PLASMA

CONCENTRATION IN PLASMA STORED AT ROOM TEMPERATURE

	CONCENTRATION (ng/ml)			
Spiked Concentration:	2.86	9.54	30.5	45.8
TIME STORED				
0 hour	2.88	9.92	30.4	46.5
1 hour	2.91	9.53	30.9	50.4
2 hour	2.73	9.80	28.4	43.5
4 hour	2.37	8.33	27.0	43.3
6 hour	2.79	8.71	25.5	42.6

TABLE 9: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON PYRIDOSTIGMINE (CATION) SPIKED HUMAN PLASMA SAMPLES@

	AT ROOM TEMPERATURE		ON ICE	
	Low Concentration	High Concentration	Low Concentration	High Concentration
Spiked Concentration	(9.54 ng/ml)	(45.8 ng/ml)	(9.54 ng/ml)	(45.8 ng/ml)
Cycle				
1	9.41	46.7	8.08	43.8
2	8.52	44.3	8.13	41.7
3	8.05	41.8	8.28	40.5
4	8.54	45.2	8.50	40.0
5	8.08	39.9	8.49	43.9

Measured concentrations are averages of two analyses.
@ Individually spiked samples.

TABLE 10: ACCURACY OF PYRIDOSTIGMINE (CATION) HUMAN PLASMA ASSAY (BLIND STUDY RESULTS)

Sample Number	Spiked Level (ng/ml)	Measured Level[#] [¥] (ng/ml)	Statistics (ng/ml)
3	0	*	Mean =
13		*	SD =
18		*	Percent CV =
28		*	Percent Bias =
6	2.71	2.74	Mean = 3.04
10		3.27	SD = 0.42
15		2.87	Percent CV = 13.78
20		2.35	Percent Bias = 12.18
24		3.15	
27		3.61	
29		3.30	
1	8.12	9.48	Mean = 8.78
4		5.46	SD = 1.59
8		8.23	Percent CV = 18.11
17		9.08	Percent Bias = 8.13
26		9.42	
30		9.52	
32		10.3	
5	18.97	18.4	Mean = 20.1
12		21.9	SD = 2.51
14		19.6	Percent CV = 12.47
21		18.4	Percent Bias = 5.96
22		19.2	
25		18.2	
31		25.0	
2	24.33	23.3	Mean = 25.10
7		26.7	SD = 1.52
9		23.3	Percent CV = 6.06
11		25.0	Percent Bias = 3.16
16		27.1	
19		24.5	
23		25.8	

[#] Measured concentrations are averages of three analyses.
[¥] Data obtained according to the method described in Study Report No. 5, dated July 21, 1986 and titled "High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma Using Silica Gel Column and an Aqueous Mobile Phase."
* = Below assay sensitivity.

LABORATORY METHODOLOGY FOR WR 242511 HUMAN AND DOG PLASMA ASSAY,* STUDY REPORT 26

A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 717 (Waters Associates, Milford, MA) or equivalent.

2. LC-600 Shimadzu Pump (Shimadzu Corp., Kyoto, Japan) or equivalent.

3. Shimadzu SPD 10A UV Detector (Shimadzu Corp., Kyoto, Japan) or equivalent.

4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

B. REAGENTS

1. WR 242511, bottle no. BM 19356 (WRAIR, Washington D.C.).

2. Chlorpheniramine (Internal Standard).

3. Triethylamine (TEA), HPLC grade, (Aldrich Chemical Co., Milwaukee. WI).

4. Acetonitrile, HPLC grade, (Fisher Scientific, Fair Lawn, NJ).

5. Phosphoric acid, reagent grade, (Fisher Scientific, Fair Lawn, NJ).

6. Sodium hydroxide, reagent grade, (Fisher Scientific, Fair Lawn, NJ).

7. Methyl *t*-butyl ether (omnisolv distilled) (EM Science, Biggstown, NJ).

8. Water, Type 1 reagent grade, (deionized by Nanopure II, Barnstead Co., Boston, MA).

*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

C. ASSAY CONDITIONS

1. DETECTOR

   Settings

   Wavelength; 240 nm
   Range: 0.003 aufs

   Lamp

   Deuterium Lamp, ABI Analytical, Inc., Ramsey, NJ.

2. COLUMN

   Axxiom Silica, 5 µm particle size, 4.6 x 250 mm (Richard Scientific, Novato, CA) or equivalent.

3. SOLVENT SYSTEM

   CH_3CN/H_2O (7:3, v/v) with 0.008% TEA and 0.005% H_3PO_4 (final concentrations)

4. FLOW RATE

   1.0 ml/min

5. STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and checked for deterioration by comparison to a newly made solution (solutions are discarded when a more than 10% change in the absolute peak height is observed or by 2 months after the preparation date).

   a. WR 242511 tartrate for interday human plasma precision expressed as the free base concentration.

Prep date: 9/21/95

Solution Type	Weight of Standard (mg)	Purity Factor*	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	14.13	0.714	50.44	50% CH_3CN	200
Control	14.10	0.714	50.33	50% CH_3CN	200

*= Molecular weights of WR 242511 free base/WR 242511 (as DL tartrate)

   b. Chlorpheniramine maleate expressed as the maleate concentration- Internal standard for interday human plasma precision.

Prep date: 9/21/95

Solution Type	Weight of Standard (mg)	Purity Factor	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	10.45	1	104.5	50% CH_3CN	100

6. WORKING SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and discarded when stock solutions were discarded or by 6 months after the preparation date).

   a. WR 242511 tartrate solutions expressed as the free base concentration.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Standard Curve	200	6.40	10	50% CH_3CN	128
Control	200	6.40	50	50% CH_3CN	128

   b. Chlorpheniramine maleate - Internal standard.

Solution Type	Conc. Diluted (µg/ml)	Volume Diluted (ml)	QS Volume (ml)	Solvent	Conc. (µg/ml)
Internal std.	100	1.00	50	50% CH_3CN	2.00

7. RETENTION TIMES (subject to change depending on temperature and column performance).

   a. WR 242511 - 10.5 min

   b. Chlorpheniramine (Internal Standard) - 15.5 min

8. BLANK PLASMA

   Human plasma (CPD or CPDA-1 as anticoagulant) was obtained from the San Francisco Irwin Memorial Blood Bank. Dog plasma (EDTA as anticoagulant) was obtained from Pel-Freez Biologicals, Rogers, AK.

9. INJECTION VOLUME

   40 µl

10. QUANTITATION

    By peak height ratio of drug peak relative to internal standard peak. Standard curves are calculated by weighted linear regression where weights = 1/y.

11. LOWER LIMIT OF QUANTITATION OF METHOD (The lower limit of quantitation of the assay of human and dog plasma for WR 242511 was based on the interday and intraday low point validation results, on standard curve calibrator results, and a minimum 3 to 1 signal to noise ratio.)

    4.00 ng/ml WR 242511 in plasma.

12. VOLUME MEASUREMENT

    Plasma sample volumes were measured with a 200 µl or a 1000 µl Gilson Pipetman. Hamilton syringes were used to measure standard and control solution volumes.

13. WISP OPERATING TEMPERATURE

    Room temperature.

14. SATURATING THE MOBILE PHASE WITH SILICA

    The mobile phase is recycled through a non analytical silica gel column overnight to saturate it with silica prior to use.

D. SAMPLE STORAGE

All samples were kept frozen at -70°C before analysis and thawed for preparation and analysis, unless specified otherwise.

E. SAMPLE PREPARATION

1. If frozen, vortex specimens for 20 seconds after sample thaws.
2. Pipet 0.5 ml of a plasma sample into a clean glass culture tube.
3. Spike standard curve sample then perform serial dilution as shown in Section G "Generation of Standard Curve Calibrators."
4. Add 0.5 ml of internal standard (2 µg/ml chlorpheniramine) solution. Vortex for 10 seconds.
5. Add 100 µl of 0.1N NaOH. Vortex for 10 seconds.
6. Add 3 ml methyl *t*-butyl ether. Vortex 1 min, twice.
7. Centrifuge 10 minutes at 3000 *g*.
8. Freeze in dry ice/methanol bath.

9. Pour supernatant into a clean glass culture tube and evaporate under N_2 at room temperature to dryness. Reconstitute in 200 µl of 70% acetonitrile.

10. Transfer to WISP vial and inject 40 µl onto HPLC column.

F. QUALITY CONTROL

1. Content and frequency of blanks

   A blank plasma sample was prepared as described in "Sample Preparation" and assayed at least once for each standard curve in precision assays.

2. PIPETTE CALIBRATION

   See SOP 2C-1.2.

3. BALANCE CALIBRATION

   See SOP 2C-2.1

G. GENERATION OF STANDARD CURVE CALIBRATORS

The generation of standard curve calibrators is described in the table below. Standard curve samples (0.5 ml) were generated by serial dilution of a 1024 ng/ml plasma sample (spiked with 40 µl of 128 µg/ml WR 242511 working solution, q.s. with plasma to 5 ml and vortexed 1 min).

Generation of WR 242511 Standard Curve Calibrators

Sample Number	Concentration in Plasma Diluted (ng/ml)	Volume Taken for Dilution (ml)	Volume of Blank Plasma Added (ml)	Standard Curve Sample Concentration (ng/ml)
9	-	-	-	1024
8	1024	1.00	1.00	512
7	512	1.00	1.00	256
6	256	1.00	1.00	128
5	128	1.00	1.00	64.0
4	64.0	1.00	1.00	32.0
3	32.0	1.00	1.00	16.0
2	16.0	1.00	1.00	8.00
1	8.00	1.00	1.00	4.00
00*	-	-	-	0
0**	-	-	-	0

*00 = Sample with no drug and no internal standard.
**0 = Sample with no drug but with internal standard.

H. GENERATION OF PRECISION SAMPLES

Interday precision samples (0.5 ml) were generated by serial dilution of a 256 ng/ml plasma sample (spiked with 10 µl of 128 µg/ml WR 242511 working solution, q.s. with plasma to 5 ml and vortexed 1 min). For intraday precision, volumes (spiking solution, q.s. plasma, taken plasma and blank plasma) were doubled.

Generation of WR 242511 Interday Precision Samples

Sample Number	Concentration in Plasma Diluted (ng/ml)	Volume Taken for Dilution (ml)	Volume of Blank Plasma Added (ml)	Standard Curve Sample Concentration (ng/ml)
Hi	-	-	-	256
Med.	256	2.00	2.00	128
Low	128	1.00	3.00	32.0
X-Lo	32.0	1.00	3.00	8.00

I. GENERATION OF RECOVERY SAMPLES

WR 242511 recovery from plasma extraction was assessed at four different concentrations by comparing the WR 242511 to internal standard peak height ratios in reference samples to the peak height ratios in plasma. Plasma (0.5 ml) samples were spiked with WR 242511 (and vortexed) then prepared as described above in "Sample Preparation," except no standard curve is used and the internal standard was added just prior to the evaporation (step 9). Reference samples were generated by preparing 0.5 ml plasma as described above in "Sample Preparation," except that WR 242511 was spiked and internal standard added to the supernatant just prior to the evaporation.

J. GENERATION OF STABILITY SAMPLES

Long term, system and bench top stability samples were generated in the same way as precision control samples. Long term stability samples were kept at -70°C or -20°C until prepared and analyzed. System stability samples were prepared as described above in "Sample Preparation," were left standing at room temperature up to 4 days, then were kept at -70°C until analyzed. Bench top stability samples were left standing at room temperature up to 6 hours then were kept at -70°C until prepared and analyzed.

The effect of repeated freeze (at -70°C) and thaw (at room temperature) cycles on stabilities of WR 242511 in human plasma samples was determined as follows: A spiked high concentration pooled biological sample (spiked with 20 µl of 128 µg/ml WR 242511 working solution, q.s. with human plasma to 10 ml and vortexed 1

min = 256 ng/ml) was diluted with blank human plasma (1.25 ml of the 256 ng/ml sample q.s. to 10 ml with blank human plasma to make 32.0 ng/ml) to make a low concentration pooled biological sample. These samples were subjected to five thaw/freeze cycles. For each cycle, a duplicate set of thaw/freeze samples (0.5 ml) was generated at each concentration. The study is run with the following procedure:

a. Prepare high and low concentration samples labeled H-1, H-2 ... H-5, and L-1, L-2 ... L-5, in duplicate.

b. Store all samples until frozen at the specified temperature.

c. Repeatedly thaw and refreeze samples according to the following table. Thaw as if for sample preparation to room temperature. Let thawed samples stand at room temperature for 1 h.

Cycle	Keep these samples in freezer	Thaw these samples
1	1	2, 3, 4, 5
2	1, 2	3, 4, 5
3	1, 2, 3	4, 5
4	1, 2, 3, 4	5
5	1, 2, 3, 4, 5	none

d. Following Cycle 5, take out all of the samples, thaw to room temperature, and assay the samples with a standard curve.

K. VALIDATION RESULTS

1. STANDARD CURVE

Chromatograms for each point in a representative standard curve for WR 242511 appear in Figure 3. Peak height ratios for these calibrators appear in Table 1. Statistical parameters of human plasma interday precision standard curve calibrators appear in Table 2. Statistical parameters of dog plasma interday precision standard curve calibrators appear in Table 11.

2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Table 3 for human plasma and Table 12 for dog plasma.

3. LLOQ

Results for this evaluation appear in Table 4 for human plasma.

4. RECOVERY
   Results for this evaluation appear in Table 5 for human plasma and Table 13 for dog plasma.

5. STABILITY

   a. System Stability: Results appear in Table 6.

   b. Long Term Stability: Results appear in Table 7.

   c. Bench Top Stability: Results appear in Table 8.

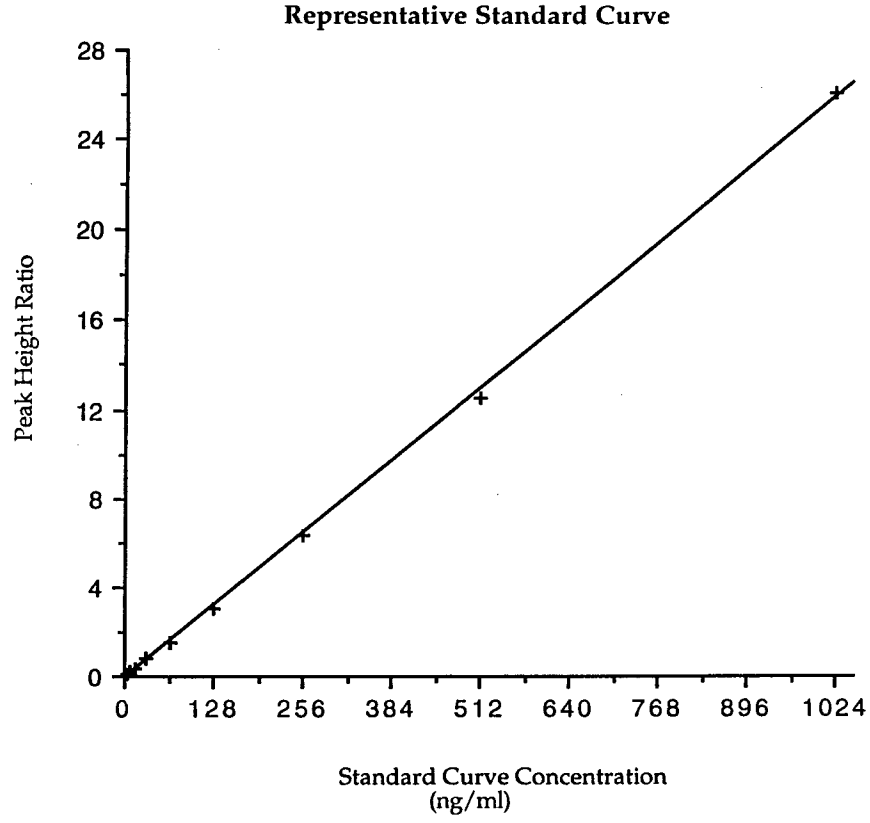
   d. Freeze/Thaw Stability: Results appear in Table 9.

6. BLIND SAMPLE ANALYSIS
   Results appear in Table 10.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR WR 242,511 PLASMA ASSAY, STUDY REPORT 26

SPIKED OR DILUTION AMOUNT (ng)*	STANDARD CURVE CONCENTRATION (ng/ml)	PEAK HEIGHT RATIO**	CALCULATED CONCENTRATION (ng/ml)
0	0	-	-
2.00	4.00	0.081	4.21
4.00	8.00	0.184	8.32
8.00	16.0	0.361	15.4
16.0	32.0	0.772	31.8
32.0	64.0	1.575	63.9
64.0	128	3.023	122
128	256	6.373	255
256	512	12.543	502
512	1024	26.073	1040

Regression equation:***
$y = 0.0250x - 0.0243$, $r^2 = 0.9995$



* In 0.5 ml of biological sample. Highest concentration sample obtained by spiking plasma with 40 µl of 128 µg/ml WR 242511 tartrate working solution (free base concentration), q.s. with plasma to 5 ml and vortexing 1 min. Remaining calibrators obtained by serial dilution.

** Ratio of drug peak height to internal standard peak height.

*** Standard curve calculated by weighted linear regression where weight = $1/y_i$.

TABLE 2: PRECISION STANDARD CURVE DATA FOR WR 242511 HUMAN PLASMA ASSAY, STUDY REPORT 26

WR 242511 Standard Curve Parameters

Validation Run Date	Validation Run No.	Slope	Intercept	Coefficient of Determination
10/7/95	6	0.02503981	-0.0243448	0.99952132
10/10/95	7	0.02212795	-0.0223938	0.99803856
10/11/95	8	0.02221112	-0.0277856	0.99812931
10/11/95	9	0.02385493	-0.0323234	0.99793302
10/12/95	10	0.02431651	-0.0284166	0.99595722
10/12/95	11	0.02419542	-0.0072980	0.99841254
12/15/95	16	0.02559090	-0.0465234	0.99922106

WR 242511 Back Calculated Standard Calibrators

Run Number	Spiked Concentration (ng/mL)								
	4.00	8.00	16.0	32.0	64.0	128	256	512	1024
	Back Calculated Concentration (ng/mL)								
6	4.21	8.32	15.4	31.8	63.9	122	255	502	1040
7	4.72	8.33	15.3	29.9	58.9	116	277	509	1030
8	4.81	8.90	15.0	28.1	57.2	132	241	511	1050
9	4.83	8.19	14.9	30.1	58.2	119	259	545	1010
10	4.29	9.31	16.6	30.3	62.4	103	249	532	1040
11	4.56	7.78	15.4	33.7	58.8	132	245	493	1060
16	4.87	7.87	15.2	29.8	58.6	124	253	509	1040
n	7	7	7	7	7	7	7	7	7
Mean	4.61	8.39	15.4	30.5	59.7	121	254	514	1040
SD	0.269	0.547	0.563	1.77	2.45	10.0	11.8	17.9	15.7
Percent CV	5.82	6.53	3.65	5.79	4.11	8.29	4.63	3.48	1.52
Percent RE	15.3	4.82	-3.75	-4.60	-6.70	-5.36	-0.725	0.474	1.42

TABLE 3: PRECISION OF WR 242511 HUMAN PLASMA ASSAY

Interday Precision WR 242511

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 8.00	32.0	128	256
		Measured Concentrations (ng/mL)			
6	1	7.84	26.2	106	224
	2	7.76	27.6	113	216
7	1	8.83	30.4	117	240
	2	8.78	27.7	107	260
8	1	8.81	28.0	121	245
	2	7.60	30.6	117	229
9	1	9.45	35.4	133	277
	2	7.89	37.0	153	270
10	1	9.35	35.5	133	274
	2	9.39	35.6	132	287
11	1	7.66	29.7	128	268
	2	7.82	31.5	125	273
n		12	12	12	12
Mean		8.43	31.3	124	255
SD		0.737	3.73	13.2	23.5
Percent CV		8.74	11.9	10.7	9.22
Percent RE		5.40	-2.29	-3.32	-0.293

Intraday Precision WR 242511

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 8.00	32.0	128	256
		Measured Concentrations (ng/mL)			
16	1	8.66	36.9	146	280
	2	9.09	33.2	138	283
	3	8.81	31.4	bc	260
	4	8.46	32.8	136	272
	5	9.16	35.1	136	270
	6	9.71	32.6	138	261
n		6	6	5	6
Mean		8.98	33.7	139	271
SD		0.443	1.99	4.15	9.47
Percent CV		4.93	5.90	2.99	3.49
Percent RE		12.3	5.21	8.44	5.86

bc = Unacceptable chromatogram.

TABLE 4: LOWER LIMIT OF QUANTITATION OF THE HUMAN PLASMA ASSAY FOR WR 242511

Spiked Concentration	4.00 ng/ml	4.00 ng/ml
	Back Calculated	Measured
Sample	Concentrations (ng/ml)	
	Interday	Intraday
1	4.21	3.69
2	4.72	3.06
3	4.81	3.33
4	4.83	3.69
5	4.29	3.15
6	4.56	3.33
Mean	4.57	3.38
SD	0.267	0.265
Percent CV	5.84	7.87
Percent RE	14.2	-15.6

TABLE 5: RECOVERY OF WR 242511 FROM HUMAN PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	PEAK HEIGHT RATIO SOLVENT	PEAK HEIGHT RATIO PLASMA	MEAN PERCENT RECOVERY
1	X Low	0.198	0.181	75.6
2		0.211	0.149	
3		bc	0.134	
Mean (± SD)		0.205 ±0.009	0.155 ±0.024	
1	Low	0.875	0.629	74.1
2		0.829	0.6454	
3		0.869	0.633	
Mean (± SD)		0.858 ±0.025	0.636 ±0.009	
1	Medium	3.458	2.629	76.4
2		3.434	2.622	
3		3.14	2.412	
Mean (± SD)		3.344 ±0.177	2.554 ±0.123	
1	High	6.248	5.090	82.3
2		5.908	5.208	
3		6.735	5.242	
Mean (± SD)		6.297 ±0.416	5.180 ±0.080	
AVERAGE =				77.1

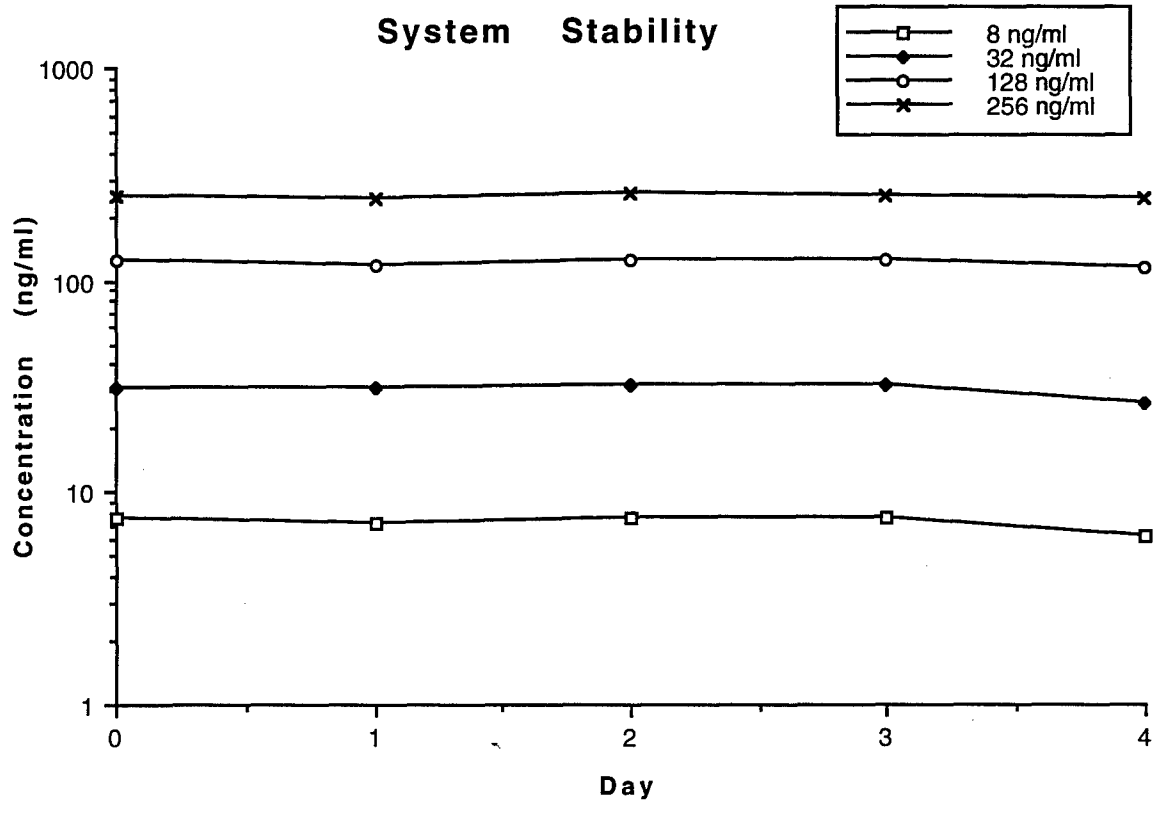
bc = Unacceptable chromatogram.

TABLE 6: SYSTEM STABILITY IN PREPARED HUMAN PLASMA

Concentration for Prepared Biological Samples Stored at Room Temperature

WR 242511

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	8.00	32.0	128	256
TIME STORED				
0 days	7.52	31.6	124	250
1 day	7.07	31.0	119	243
2 days	7.64	32.0	124	258
3 days	7.66	32.2	126	252
4 days	6.25	26.1	113	242

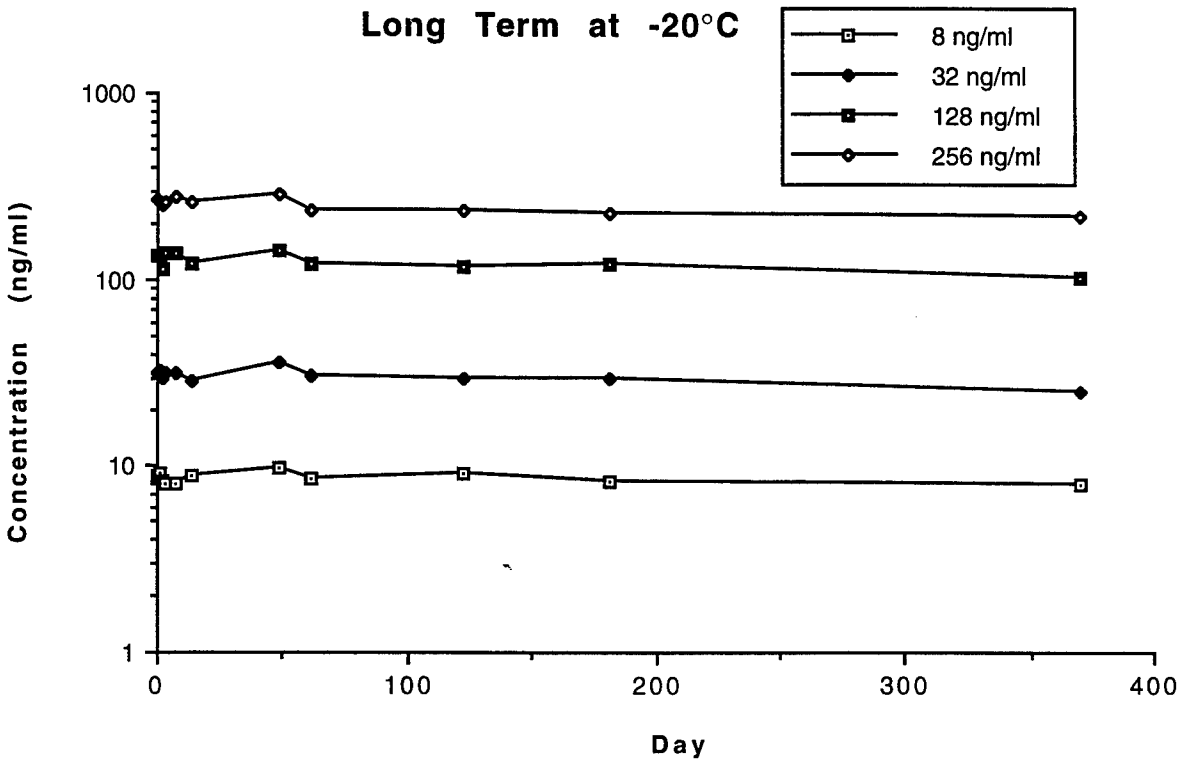


* Measured concentrations are averages of two analyses.

TABLE 7A: LONG TERM STABILITY OF WR 242511 IN HUMAN PLASMA

WR 242511 CONCENTRATION IN PLASMA STORED AT -20°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	8.00	32.0	128	256
TIME STORED				
0 days	8.63	32.0	136	265
1 day	9.01	32.6	136	260
2 days	8.14	29.7	113	250
3 days	8.01	31.2	139	256
1 week	7.89	31.4	138	275
2 weeks	8.80	28.8	120	262
49 days	9.74	36.0	145	291
2 months	8.58	30.5	120	236
4 months	8.94	29.7	116	237
6 months	8.24	29.2	121	227
1 year	7.92	25.4	103	221

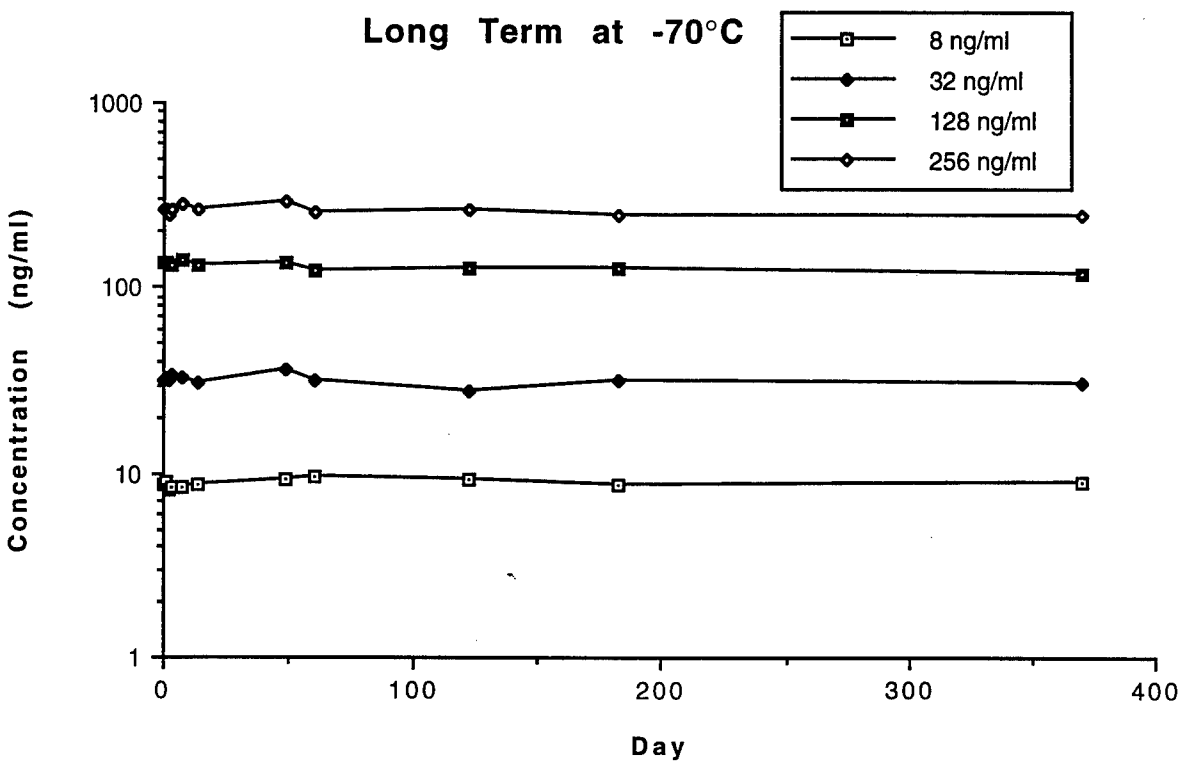


* Measured concentrations are averages of two analyses.

TABLE 7B: LONG TERM STABILITY OF WR 242511 IN HUMAN PLASMA

WR 242511 CONCENTRATION IN PLASMA STORED AT -70°C

	CONCENTRATION* (ng/ml)			
Spiked Concentration:	8.00	32.0	128	256
TIME STORED				
0 days	8.63	32.0	136	265
1 day	9.09	32.7	135	263
2 days	8.16	31.7	132	249
3 days	8.45	33.5	134	264
1 week	8.42	32.3	139	285
2 weeks	8.78	31.0	130	261
49 days	9.31	36.7	135	290
2 month	9.64	31.9	122	253
4 months	9.16	28.0	127	261
6 months	8.73	31.4	127	249
1 year	8.82	30.8	118	244

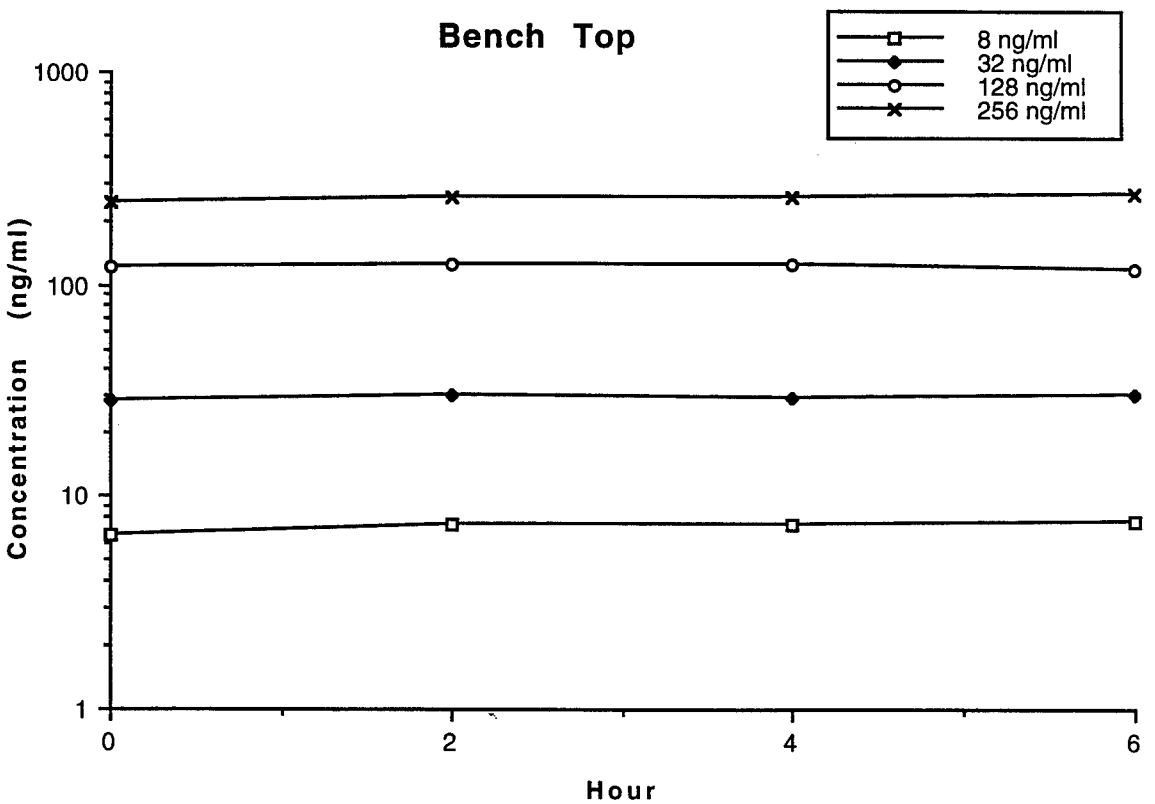


* Measured concentrations are averages of two analyses.

TABLE 8: BENCH TOP STABILITY OF WR 242511 IN SPIKED HUMAN PLASMA

CONCENTRATION IN PLASMA STORED AT ROOM TEMPERATURE

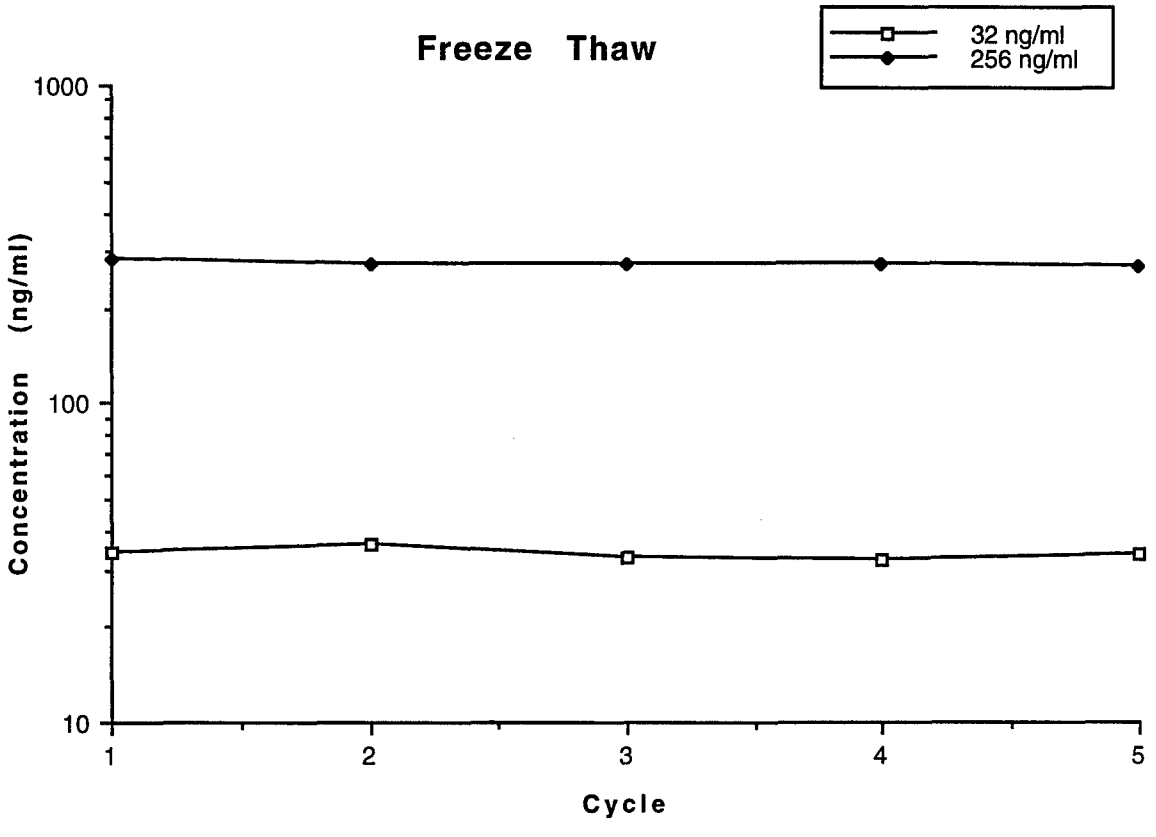
	CONCENTRATION (ng/ml)			
Spiked Concentration:	8.00	32.0	128	256
TIME STORED				
0 hour	6.63	28.7	120	240
2 hour	7.36	30.6	126	257
4 hour	7.29	29.4	124	259
6 hour	7.62	30.0	118	265



Measured concentrations are averages of two analyses.

TABLE 9: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON WR 242511 SPIKED HUMAN PLASMA SAMPLES@

	AT ROOM TEMPERATURE	
	Low Concentration	High Concentration
Spiked Concentration	(32.0 ng/ml)	(256 ng/ml)
Cycle		
1	34.4	284
2	36.4	274
3	32.8	277
4	32.2	275
5	33.5	271



@ Individually spiked samples.
# Measured concentrations are averages of two analyses.

TABLE 10: ACCURACY OF WR 242511 HUMAN PLASMA ASSAY (BLIND STUDY RESULTS)

Sample Number	Spiked Level (ng/ml)	Measured Level# (ng/ml)	Statistics (ng/ml)
4	0	6.48@	
9		*	
13		*	
23		*	
30		*	
7	4.70	5.93	Mean = 5.73
15		6.39	SD = 0.55
20		5.13	Percent CV = 9.61
24		5.46	Percent RE = 21.9
27		*@	
1	25.9	26.7	Mean = 27.0
3		26.6	SD = 0.635
11		26.6	Percent CV = 2.36
17		43.0@	Percent RE = 4.05
29		27.9	
5	112	103	Mean = 103
10		103	SD = 1.82
18		102	Percent CV = 1.77
21		105	Percent RE = -8.39
28		100	
6	415	390	Mean = 389
8		392	SD = 7.81
12		397	Percent CV = 2.01
19		390	Percent RE = -6.27
26		376	
2	822	793	Mean = 793
14		802	SD = 7.60
16		791	Percent CV = 0.958
22		782	Percent RE = -3.50
25		798	

Measured concentrations are averages of three analyses.
@ Anomalous result: samples 4 and 27 appear to have been switched or mislabeled and sample 17 has bad precision. Results for these three samples were not included in CV and RE calculations.
* = Below assay sensitivity.

TABLE 11: PRECISION STANDARD CURVE DATA FOR WR 242511 DOG PLASMA ASSAY, STUDY REPORT 26

WR 242511 Standard Curve Parameters

Validation Run Date	Validation Run No.	Slope	Intercept	Coefficient of Determination
9/4/96	inter1	0.02111344	-0.0295347	0.99857702
9/5/96	intra	0.02444799	-0.0069828	0.99891019
9/12/96	inter2	0.02755975	-0.0016602	0.99275586
9/13/96	inter3	0.02671813	-0.0111891	0.99343441

WR 242511 Back Calculated Standard Calibrators

Run Number	Spiked Concentration (ng/mL)								
	4.00	8.00	16.0	32.0	64.0	128	256	512	1024
	Back Calculated Concentration (ng/mL)								
inter1	4.76	8.50	14.4	29.6	57.8	134	251	498	1050
intra	4.25	8.30	16.2	32.1	63.0	120	245	503	1050
inter2	4.12	8.22	14.9	42.3	61.8	114	242	471	1100
inter3	4.54	7.94	14.6	41.0	61.5	112	242	477	1090
n	4	4	4	4	4	4	4	4	4
Mean	4.42	8.24	15.0	36.3	61.0	120	245	487	1070
SD	0.288	0.232	0.81	6.34	2.25	9.93	4.24	15.6	26.3
Percent CV	6.52	2.82	5.39	17.5	3.68	8.28	1.73	3.21	2.45
Percent RE	10.4	3.00	-6.09	13.3	-4.65	-6.25	-4.30	-4.83	4.74

TABLE 12: PRECISION OF WR 242511 DOG PLASMA ASSAY

Interday Precision WR 242511

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 8.00	32.0	128	256
		Measured Concentrations (ng/mL)			
inter1	1	9.02	30.5	115	224
	2	8.60	30.6	123	262
inter2	1	8.51	29.2	133	250
	2	6.23	31.9	123	234
inter3	1	8.43	28.3	134	252
	2	7.16	30.4	122	238
	n	6	6	6	6
	Mean	7.99	30.2	125	243
	SD	1.07	1.25	7.24	13.8
	Percent CV	13.3	4.14	5.79	5.69
	Percent RE	-0.104	-5.78	-2.34	-4.95

Intraday Precision WR 242511

Validation Run No.	QC Sample No.	Spiked Concentrations (ng/mL) 8.00	32.0	128	256
		Measured Concentrations (ng/mL)			
intra	1	7.20	27.9	114	231
	2	8.14	29.0	115	231
	3	7.61	29.1	113	233
	4	8.26	28.9	116	229
	5	7.57	28.8	114	228
	6	7.61	28.5	113	231
	n	6	6	6	6
	Mean	7.73	28.7	114	231
	SD	0.396	0.443	1.17	1.76
	Percent CV	5.12	1.54	1.02	0.764
	Percent RE	-3.35	-10.3	-10.8	-9.96

TABLE 13: RECOVERY OF WR 242511 FROM DOG PLASMA

SAMPLE ID	SPIKED CONCENTRATION Range	PEAK HEIGHT RATIO		MEAN PERCENT RECOVERY
		SOLVENT	PLASMA	
1	X Low	0.140	0.114	73.9
2		0.206	0.134	
3		0.129	0.103	
Mean (± SD)		0.158 ±0.042	0.117 ±0.016	
1	Low	0.541	0.510	87.4
2		0.521	0.495	
3		0.563	0.415	
Mean (± SD)		0.542 ±0.021	0.473 ±0.051	
1	Medium	2.279	2.008	75.2
2		3.069	1.871	
3		2.484	2.011	
Mean (± SD)		2.611 ±0.410	1.963 ±0.080	
1	High	5.261	4.022	80.8
2		5.943	4.578	
3		4.727	4.278	
Mean (± SD)		5.310 ±0.609	4.293 ±0.278	
AVERAGE =				79.3